

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>C07H 21/02, 21/04, C12P 19/34, C12Q 1/68</b>		<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 96/34876</b>
			<b>(43) International Publication Date:</b> 7 November 1996 (07.11.96)
<b>(21) International Application Number:</b> PCT/US96/06171		<b>(74) Agents:</b> SWANSON, Barry, J. et al.; Swanson & Bratschun, L.L.C., Suite 200, 8400 East Prentice Avenue, Englewood, CO 80111 (US).	
<b>(22) International Filing Date:</b> 2 May 1996 (02.05.96)			
<b>(30) Priority Data:</b> 08/434,465 4 May 1995 (04.05.95) US 08/464,443 5 June 1995 (05.06.95) US		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).	
<b>(60) Parent Applications or Grants</b> <b>(63) Related by Continuation</b> US 08/434,465 (CIP) Filed on 4 May 1995 (04.05.95) US 08/464,443 (CIP) Filed on 5 June 1995 (05.06.95)		<b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
<b>(71) Applicant (for all designated States except US):</b> NEXSTAR PHARMACEUTICALS, INC. [US/US]; Suite 200, 2860 Wilderness Place, Boulder, CO 80301 (US).			
<b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> GOLD, Larry [US/US]; 1033 5th Street, Boulder, CO 80302 (US). SCHMIDT, Paul, G. [US/US]; 1730 Euclid Avenue, San Marino, CA 91108 (US). JANJIC, Nebojsa [US/US]; 6973 Carter Trail, Boulder, CO 80301 (US).			
<b>(54) Title:</b> NUCLEIC ACID LIGAND COMPLEXES			
<b>(57) Abstract</b> <p>This invention discloses a method for preparing a therapeutic or diagnostic complex comprised of a nucleic acid ligand and a lipophilic compound or non-immunogenic, high molecular weight compound by identifying a nucleic acid ligand by SELEX methodology and associating the nucleic acid ligand with a lipophilic compound or a non-immunogenic, high molecular weight compound. The invention further discloses complexes comprising one or more nucleic acid ligands in association with a lipophilic compound or non-immunogenic, high molecular weight compound.</p>			

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

## NUCLEIC ACID LIGAND COMPLEXES

### FIELD OF THE INVENTION

This invention relates to a method for preparing a therapeutic or diagnostic Complex comprised of a Nucleic Acid Ligand and a Lipophilic Compound or Non-Immunogenic, High Molecular Weight Compound by identifying a Nucleic Acid Ligand by SELEX methodology and associating the Nucleic Acid Ligand with a Lipophilic Compound or a Non-Immunogenic, High Molecular Weight Compound. The invention further relates to improving the pharmacokinetic properties of a Nucleic Acid Ligand by associating the Nucleic Acid Ligand to a Lipophilic Compound or Non-Immunogenic, High Molecular Weight Compound to form a Complex. The invention further relates to a method for targeting a therapeutic or diagnostic agent to a specific predetermined biological Target by associating the agent with a Complex comprised of a Nucleic Acid Ligand and a Lipophilic Compound or Non-Immunogenic, High Molecular Weight Compound, wherein the Nucleic Acid Ligand has a SELEX Target associated with the specific predetermined Target and the Nucleic Acid Ligand is associated with the exterior of the Complex. The invention also includes complexes comprising one or more Nucleic Acid Ligand in association with a Lipophilic Compound or Non-Immunogenic, High Molecular Weight Compound.

### 20 BACKGROUND OF THE INVENTION

#### A. SELEX

The dogma for many years was that nucleic acids had primarily an informational role. Through a method known as Systematic Evolution of Ligands by EXponential enrichment, termed SELEX, it has become clear that nucleic acids have three dimensional structural diversity not unlike proteins. SELEX is a method for the *in vitro* evolution of nucleic acid molecules with highly specific binding to target molecules and is described in United States Patent Application Serial No. 07/536,428, filed June 11, 1990, entitled "Systematic Evolution of Ligands by Exponential Enrichment", now abandoned, United States Patent Application Serial No. 07/714,131, filed June 10, 1991, entitled "Nucleic Acid Ligands", now United States Patent No. 5,475,096, United States Patent Application Serial No. 07/931,473, filed August 17, 1992, entitled "Nucleic Acid Ligands", now United States Patent No. 5,270,163 (see also PCT/US91/04078), each of which is specifically incorporated by reference herein. Each of these applications, collectively referred to herein

as the SELEX Patent Applications, describes a fundamentally novel method for making a Nucleic Acid Ligand to any desired target molecule. The SELEX process provides a class of products which are referred to as Nucleic Acid Ligands, each ligand having a unique sequence, and which has the property of binding specifically to a desired target compound or molecule. Each SELEX-identified Nucleic Acid Ligand is a specific ligand of a given target compound or molecule. SELEX is based on the unique insight that Nucleic Acids have sufficient capacity for forming a variety of two- and three-dimensional structures and sufficient chemical versatility available within their monomers to act as ligands (form specific binding pairs) with virtually any chemical compound, whether monomeric or polymeric. Molecules of any size or composition can serve as targets.

The SELEX method involves selection from a mixture of candidate oligonucleotides and step-wise iterations of binding, partitioning and amplification, using the same general selection scheme, to achieve virtually any desired criterion of binding affinity and selectivity. Starting from a mixture of Nucleic Acids, preferably comprising a segment of randomized sequence, the SELEX method includes steps of contacting the mixture with the target under conditions favorable for binding, partitioning unbound Nucleic Acids from those Nucleic Acids which have bound specifically to target molecules, dissociating the Nucleic Acid-target complexes, amplifying the Nucleic Acids dissociated from the Nucleic Acid-target complexes to yield a ligand-enriched mixture of Nucleic Acids, then reiterating the steps of binding, partitioning, dissociating and amplifying through as many cycles as desired to yield highly specific high affinity Nucleic Acid Ligands to the target molecule.

It has been recognized by the present inventors that the SELEX method demonstrates that Nucleic Acids as chemical compounds can form a wide array of shapes, sizes and configurations, and are capable of a far broader repertoire of binding and other functions than those displayed by Nucleic Acids in biological systems.

The present inventors have recognized that SELEX or SELEX-like processes could be used to identify Nucleic Acids which can facilitate any chosen reaction in a manner similar to that in which Nucleic Acid Ligands can be identified for any given target. In theory, within a Candidate Mixture of approximately  $10^{13}$  to  $10^{18}$  Nucleic Acids, the present inventors postulate that at least one Nucleic Acid exists with the appropriate shape to facilitate each of a broad variety of physical and chemical interactions.

The basic SELEX method has been modified to achieve a number of specific objectives. For example, United States Patent Application Serial No. 07/960,093, filed



October 14, 1992, entitled "Method for Selecting Nucleic Acids on the Basis of Structure," describes the use of SELEX in conjunction with gel electrophoresis to select Nucleic Acid molecules with specific structural characteristics, such as bent DNA. United States Patent Application Serial No. 08/123,935, filed September 17, 1993, entitled "Photoselection of Nucleic Acid Ligands," describes a SELEX based method for selecting Nucleic Acid Ligands containing photoreactive groups capable of binding and/or photocrosslinking to and/or photoinactivating a target molecule. United States Patent Application Serial No. 08/134,028, filed October 7, 1993, entitled "High-Affinity Nucleic Acid Ligands That Discriminate Between Theophylline and Caffeine," describes a method for identifying highly specific Nucleic Acid Ligands able to discriminate between closely related molecules, which can be non-peptidic, termed Counter-SELEX. United States Patent Application Serial No. 08/143,564, filed October 25, 1993, entitled "Systematic Evolution of Ligands by EXponential Enrichment: Solution SELEX," describes a SELEX-based method which achieves highly efficient partitioning between oligonucleotides having high and low affinity for a target molecule.

The SELEX method encompasses the identification of high-affinity Nucleic Acid Ligands containing modified nucleotides conferring improved characteristics on the ligand, such as improved *in vivo* stability or improved delivery characteristics. Examples of such modifications include chemical substitutions at the ribose and/or phosphate and/or base positions. SELEX-identified Nucleic Acid Ligands containing modified nucleotides are described in United States Patent Application Serial No. 08/117,991, filed September 8, 1993, entitled "High Affinity Nucleic Acid Ligands Containing Modified Nucleotides," that describes oligonucleotides containing nucleotide derivatives chemically modified at the 5- and 2'-positions of pyrimidines. United States Patent Application Serial No. 08/134,028, *supra*, describes highly specific Nucleic Acid Ligands containing one or more nucleotides modified with 2'-amino (2'-NH<sub>2</sub>), 2'-fluoro (2'-F), and/or 2'-O-methyl (2'-OMe). United States Patent Application Serial No. 08/264,029, filed June 22, 1994, entitled "Novel Method of Preparation of 2' Modified Pyrimidine Intramolecular Nucleophilic Displacement", describes oligonucleotides containing various 2'-modified pyrimidines.

The SELEX method encompasses combining selected oligonucleotides with other selected oligonucleotides and non-oligonucleotide functional units as described in United States Patent Application Serial No. 08/284,063, filed August 2, 1994, entitled

"Systematic Evolution of Ligands by Exponential Enrichment: Chimeric SELEX" and United States Patent Application Serial No. 08/234,997, filed April 28, 1994, entitled "Systematic Evolution of Ligands by Exponential Enrichment: Blended SELEX," respectively. These applications allow the combination of the broad array of shapes and other properties, and the efficient amplification and replication properties, of oligonucleotides with the desirable properties of other molecules. Each of the above described patent applications which describe modifications of the basic SELEX procedure are specifically incorporated by reference herein in their entirety.

#### B. LIPID CONSTRUCTS

10 Lipid Bilayer Vesicles are closed, fluid-filled microscopic spheres which are formed principally from individual molecules having polar (hydrophilic) and non-polar (lipophilic) portions. The hydrophilic portions may comprise phosphato, glycerylphosphato, carboxy, sulfato, amino, hydroxy, choline or other polar groups. Examples of lipophilic groups are saturated or unsaturated hydrocarbons such as alkyl, alkenyl or other lipid groups. Sterols (e.g., cholesterol) and other pharmaceutically acceptable adjuvants (including anti-oxidants like alpha-tocopherol) may also be included to improve vesicle stability or confer other desirable characteristics.

Liposomes are a subset of these bilayer vesicles and are comprised principally of phospholipid molecules that contain two hydrophobic tails consisting of fatty acid chains. Upon exposure to water, these molecules spontaneously align to form spherical, bilayer membranes with the lipophilic ends of the molecules in each layer associated in the center of the membrane and the opposing polar ends forming the respective inner and outer surface of the bilayer membrane(s). Thus, each side of the membrane presents a hydrophilic surface while the interior of the membrane comprises a lipophilic medium. These membranes may be arranged in a series of concentric, spherical membranes separated by thin strata of water, in a manner not dissimilar to the layers of an onion, around an internal aqueous space. These multilamellar vesicles (MLV) can be converted into small or Unilamellar Vesicles (UV), with the application of a shearing force.

The therapeutic use of liposomes includes the delivery of drugs which are normally toxic in the free form. In the liposomal form, the toxic drug is occluded, and may be directed away from the tissues sensitive to the drug and targeted to selected areas. Liposomes can also be used therapeutically to release drugs over a prolonged period of

time, reducing the frequency of administration. In addition, liposomes can provide a method for forming aqueous dispersions of hydrophobic or amphiphilic drugs, which are normally unsuitable for intravenous delivery.

In order for many drugs and imaging agents to have therapeutic or diagnostic potential, it is necessary for them to be delivered to the proper location in the body, and the liposome can thus be readily injected and form the basis for sustained release and drug delivery to specific cell types, or parts of the body. Several techniques can be employed to use liposomes to target encapsulated drugs to selected host tissues, and away from sensitive tissues. These techniques include manipulating the size of the liposomes, their net surface charge, and their route of administration. MLVs, primarily because they are relatively large, are usually rapidly taken up by the reticuloendothelial system (principally the liver and spleen). UVs, on the other hand, have been found to exhibit increased circulation times, decreased clearance rates and greater biodistribution relative to MLVs.

Passive delivery of liposomes involves the use of various routes of administration, e.g., intravenous, subcutaneous, intramuscular and topical. Each route produces differences in localization of the liposomes. Two common methods used to direct liposomes actively to selected target areas involve attachment of either antibodies or specific receptor ligands to the surface of the liposomes. Antibodies are known to have a high specificity for their corresponding antigen and have been attached to the surface of liposomes, but the results have been less than successful in many instances. Some efforts, however, have been successful in targeting liposomes to tumors without the use of antibodies, see, for example, U.S. Patent No. 5,019,369.

An area of development aggressively pursued by researchers is the delivery of agents not only to a specific cell type but into the cell's cytoplasm and, further yet, into the nucleus. This is particularly important for the delivery of biological agents such as DNA, RNA, ribozymes and proteins. A promising therapeutic pursuit in this area involves the use of antisense DNA and RNA oligonucleotides for the treatment of disease. However, one major problem encountered in the effective application of antisense technology is that oligonucleotides in their phosphodiester form are quickly degraded in body fluids and by intracellular and extracellular enzymes, such as endonucleases and exonucleases, before the target cell is reached. Intravenous administration also results in rapid clearance from the bloodstream by the kidney, and uptake is insufficient to produce an effective intracellular drug concentration. Liposome encapsulation protects the

oligonucleotides from the degradative enzymes, increases the circulation half-life and increases uptake efficiency as a result of phagocytosis of the Liposomes. In this way, oligonucleotides are able to reach their desired target and to be delivered to cells *in vivo*.

A few instances have been reported where researchers have attached antisense oligonucleotides to Lipophilic Compounds or Non-Immunogenic, High Molecular Weight Compounds. Antisense oligonucleotides, however, are only effective as intracellular agents. Antisense oligodeoxyribonucleotides targeted to the epidermal growth factor (EGF) receptor have been encapsulated into Liposomes linked to folate via a polyethylene glycol spacer (folate-PEG-Liposomes) and delivered into cultured KB cells via folate receptor-mediated endocytosis (Wang *et al.* (1995) 92:3318-3322). In addition, a Lipophilic Compound covalently attached to an antisense oligonucleotide has been demonstrated in the literature (EP 462 145 B1).

#### **SUMMARY OF THE INVENTION**

The present invention provides a method for preparing a therapeutic or diagnostic Complex comprised of a Nucleic Acid Ligand and a Lipophilic Compound or Non-Immunogenic, High Molecular Weight Compound by the method comprising identifying a Nucleic Acid Ligand from a Candidate Mixture of Nucleic Acids where the Nucleic Acid is a ligand of a given target by the method of (a) contacting the Candidate Mixture of Nucleic Acids with the target, (b) partitioning between members of said Candidate Mixture on the basis of affinity to the target, and c) amplifying the selected molecules to yield a mixture of Nucleic Acids enriched for Nucleic Acid sequences with a relatively higher affinity for binding to the target, and associating said identified Nucleic Acid Ligand with a Lipophilic Compound or a Non-Immunogenic, High Molecular Weight Compound.

In another embodiment, this invention provides a method for improving the cellular uptake of a Nucleic Acid Ligand having an intracellular SELEX Target by associating the Nucleic Acid Ligand with a Lipophilic Compound or a Non-Immunogenic, High Molecular Weight Compound to form a Complex comprised of a Nucleic Acid Ligand and a Lipophilic Compound or a Non-Immunogenic, High Molecular Weight Compound and administering the Complex to a patient.

In another embodiment, this invention provides a method for improving the pharmacokinetic properties of a Nucleic Acid Ligand by associating the Nucleic Acid Ligand to a Lipophilic Compound or a Non-Immunogenic, High Molecular Weight

Compound to form a Complex comprised of a Nucleic Acid Ligand and a Lipophilic Compound or a Non-Immunogenic, High Molecular Weight Compound and administering the Complex to a patient.

5 In another embodiment, this invention provides a method for targeting a therapeutic or diagnostic agent to a specific predetermined biological Target in a patient comprising associating the therapeutic or diagnostic agent with a Complex comprised of a Nucleic Acid Ligand and a Lipophilic Compound or Non-Immunogenic, High Molecular Weight Compound, wherein the Nucleic Acid Ligand has a SELEX Target associated with the specific predetermined Target, and the Nucleic Acid Ligand is associated with the  
10 exterior of the Complex and administering the Complex to a patient.

It is an object of the present invention to provide Complexes comprising one or more Nucleic Acid Ligands in association with a Lipophilic Compound or Non-Immunogenic, High Molecular Weight Compound and methods for producing the same. It is a further object of the invention to provide one or more Nucleic Acid Ligands in  
15 association with a Lipophilic Compound or Non-Immunogenic, High Molecular Weight Compound with Improved Pharmacokinetic Properties. In another aspect of the invention, the Lipophilic Compound is a Lipid Construct. In this embodiment, the Lipid Construct is preferably a Lipid Bilayer Vesicle and most preferably a Liposome. In certain embodiments of the invention the Lipophilic Compound is cholesterol, dialkyl  
20 glycerol, or diacyl glycerol. In another embodiment of the invention, the Non-Immunogenic, High Molecular Weight Compound is PEG. In another embodiment of the invention, the Non-Immunogenic, High Molecular Weight Compound is magnetite. In the preferred embodiment, the Nucleic Acid Ligand is identified according to the SELEX method.

25 In embodiments of the invention directed to Complexes comprising cholesterol, dialkyl glycerol, diacyl glycerol, PEG, or magnetite in association with a Nucleic Acid Ligand or ligands, the Nucleic Acid Ligand or ligands can serve in a targeting capacity.

In embodiments of the invention directed to Complexes comprising a Lipid Construct where the Lipid Construct is of a type that has a membrane defining an interior  
30 compartment such as a Lipid Bilayer Vesicle, the Nucleic Acid Ligand in association with the Lipid Construct may be associated with the membrane of the Lipid Construct or encapsulated within the compartment. In embodiments where the Nucleic Acid Ligand is in association with the membrane, the Nucleic Acid Ligand can associate with the interior-

facing or exterior-facing part of the membrane, such that the Nucleic Acid Ligand is projecting in to or out of the vesicle. In embodiments where the Nucleic Acid Ligand is projecting out of the Complex, the Nucleic Acid Ligand can serve in a targeting capacity. Non-Immunogenic, High Molecular Weight Compounds can also be associated with the membrane. In one embodiment, the Nucleic Acid Ligand may be associated with a Non-Immunogenic, High Molecular Weight Compound which is associated with the membrane. The membrane may have associated with it additional Non-Immunogenic, High Molecular Weight Compounds not associated with a Nucleic Acid Ligand.

In embodiments where the Nucleic Acid Ligand of the Complex serves in a targeting capacity, the Complex can incorporate or have associated with it therapeutic or diagnostic agents. In one embodiment, the therapeutic agent is a drug. In an alternative embodiment, the therapeutic or diagnostic agent is one or more additional Nucleic Acid Ligands. Nucleic Acid Ligands specific for different targets can project from the external surface of the Complex. The Complex can project from the external surface one or more Nucleic Acid Ligands which are specific for different SELEX Targets on the same Target.

These and other objects, as well as the nature, scope and utilization of this invention, will become readily apparent to those skilled in the art from the following description and the appended claims.

## **BRIEF DESCRIPTION OF THE FIGURES**

Figures 1A-1Y show the molecular descriptions of NX229, NX232, NX253, NX256, 225T3, 225T3N, T-P4, NX-256-PEG-20,000, 225T3N-PEG-3400, T-P4-PEG-(20,000 or 10,000), NX268, NX191, JW966, NX278, JW986, NX213, NX244, JW1130, NX287, JW1336-20K PEG, JW1379, JW1380, scNX278, JW986-PEG-(10,000, 20,000, or 40,000), and JW1336 (SEQ ID NOS:6-30). A lower case letter preceding a nucleotide indicates the following: m=2'-O-Methyl, a=2'-amino, r=ribo, and f=2'-fluoro. No letter preceding a nucleotide indicates a deoxyribonucleotide (2'H). An S following a nucleotide denotes a backbone modification consisting of a phosphorothioate internucleoside linkage.

Figure 2 shows gel permeation chromatograms for empty Liposomes (Empty), Liposomes with 4.7 mg NX232 (L-NeX2a), Liposomes with 11.8 mg NX232 (L-NeX2b), free NX232 at 72 mg (Free 72 mg), free NX232 at 7.2 mg (Free 7.2 mg), and free NX232 at 10 mg which had been sonicated (Free/soni 10 mg).

Figure 3 summarizes the data for the plasma concentration of NX229, NX232, NX253, NX253 + Liposome, and NX256-PEG20K as a function of time following the bolus injection.

Figure 4 summarizes the data for the plasma concentration of NX213 (SEQ ID NO:21), NX268 (SEQ ID NO:16), NX278 (SEQ ID NO:19), NX278 + liposome, JW986 (SEQ ID NO:20), NX213 liposome encapsulated, and NX244 (SEQ ID NO:22) as a function of time following the bolus injection.

Figure 5 summarizes the data for the plasma concentration of JW966 (SEQ ID NO:18) and JW966 + liposome as a function of time following the bolus injection.

Figure 6 summarizes the data for the plasma concentration of NX268 (SEQ ID NO:16) and NX268 + liposome as a function of time following the bolus injection.

Figure 7 summarizes the data for the plasma concentration of NX191 (SEQ ID NO:17), JW986 + PEG20K, PEG40K, and PEG10K (SEQ ID NO:29) as a function of time following the bolus injection.

Figure 8 summarizes the data for the plasma concentration of JW986 + PEG20K (SEQ ID NO:29) and JW1130 (SEQ ID NO:23) as a function of time following the bolus injection.

Figure 9 summarizes the data for the plasma concentration of NX287 + PEG40K (SEQ ID NO:24) and NX256 (SEQ ID NO:9) as a function of time following the bolus injection.

Figure 10 summarizes the data for the plasma concentration of JW1130 (SEQ ID NO:23), 1136-PEG20K (SEQ ID NO:25), 1336 (SEQ ID NO:30), and 1379/80 (SEQ ID NOS:26-27) as a function of time following the bolus injection.

Figure 11 shows the chromatograms for NX232 (SEQ ID NO:7), NX232 + 1% PGSUV, NX232 + 2.5% PGSUV, and NX232 + PGSUV.

Figure 12 shows the fraction of bound Nucleic Acid Ligand (NX253) (SEQ ID NO:8) as a function of Liposome:Nucleic Acid Ligand ratio.

**DETAILED DESCRIPTION OF THE INVENTION****DEFINITIONS:**

"**Covalent Bond**" is the chemical bond formed by the sharing of electrons.

5 "Non-Covalent Interactions" are means of holding together molecular entities by interactions other than Covalent Bonds including ionic interactions and hydrogen bonds.

10 "Lipid Constructs," for purposes of this invention, are structures containing lipids, phospholipids, or derivatives thereof comprising a variety of different structural arrangements which lipids are known to adopt in aqueous suspension. These structures include, but are not limited to, Lipid Bilayer Vesicles, micelles, Liposomes, emulsions, lipid ribbons or sheets, and may be complexed with a variety of drugs and adjuvants which are known to be pharmaceutically acceptable. Common adjuvants include cholesterol and alpha-tocopherol, among others. The Lipid Constructs may be used alone or in any combination which one skilled in the art would appreciate to provide the characteristics  
15 desired for a particular application. In addition, the technical aspects of Lipid Constructs and Liposome formation are well known in the art and any of the methods commonly practiced in the field may be used for the present invention.

20 "Lipophilic Compounds" are compounds which have the propensity to associate with or partition into lipid and/or other materials or phases with low dielectric constants, including structures that are comprised substantially of lipophilic components. Lipophilic Compounds include Lipid Constructs as well as non-lipid containing compounds that have the propensity to associate with lipid (and/or other materials or phases with low dielectric constants). Cholesterol, phospholipid, and dialkyl glycerol are further examples of Lipophilic Compounds.

25 "Complex" as used herein describes the molecular entity formed by the association of a Nucleic Acid Ligand with a Lipophilic Compound or Non-Immunogenic, High Molecular Weight Compound. The association can be through either Covalent Bonds or Non-Covalent Interactions.

30 "Nucleic Acid Ligand" as used herein is a non-naturally occurring Nucleic Acid having a desirable action on a SELEX Target. A desirable action includes, but is not limited to, binding of the SELEX Target, catalytically changing the SELEX Target, reacting with the SELEX Target in a way which modifies/alters the SELEX Target or the functional activity of the SELEX Target, covalently attaching to the SELEX Target as in a suicide



inhibitor, facilitating the reaction between the Target and another molecule. In the preferred embodiment, the action is specific binding affinity for a Target molecule, such Target molecule being a three dimensional chemical structure other than a polynucleotide that binds to the Nucleic Acid Ligand through a mechanism which predominantly depends on Watson/Crick base pairing or triple helix binding, wherein the Nucleic Acid Ligand is not a Nucleic Acid having the known physiological function of being bound by the Target molecule. In preferred embodiments of the invention, the Nucleic Acid Ligand of the Complexes of the invention are identified by the SELEX methodology. Nucleic Acid Ligands include Nucleic Acids that are identified from a Candidate Mixture of Nucleic Acids, said Nucleic Acid being a ligand of a given Target, by the method comprising a) contacting the Candidate Mixture with the Target, wherein Nucleic Acids having an increased affinity to the Target relative to the Candidate Mixture may be partitioned from the remainder of the Candidate Mixture; b) partitioning the increased affinity Nucleic Acids from the remainder of the Candidate Mixture; and c) amplifying the increased affinity Nucleic Acids to yield a ligand-enriched mixture of Nucleic Acids.

"**Candidate Mixture**" is a mixture of Nucleic Acids of differing sequence from which to select a desired ligand. The source of a Candidate Mixture can be from naturally-occurring Nucleic Acids or fragments thereof, chemically synthesized Nucleic Acids, enzymatically synthesized Nucleic Acids or Nucleic Acids made by a combination of the foregoing techniques. In a preferred embodiment, each Nucleic Acid has fixed sequences surrounding a randomized region to facilitate the amplification process.

"**Nucleic Acid**" means either DNA, RNA, single-stranded or double-stranded and any chemical modifications thereof. Modifications include, but are not limited to, those which provide other chemical groups that incorporate additional charge, polarizability, hydrogen bonding, electrostatic interaction, and fluxionality to the Nucleic Acid Ligand bases or to the Nucleic Acid Ligand as a whole. Such modifications include, but are not limited to, 2'-position sugar modifications, 5-position pyrimidine modifications, 8-position purine modifications, modifications at exocyclic amines, substitution of 4-thiouridine, substitution of 5-bromo or 5-iodo-uracil, backbone modifications such as internucleoside phosphorothioate linkages, methylations, unusual base-pairing combinations such as the isobases isocytidine and isoguanidine and the like. Modifications can also include 3' and 5' modifications such as capping.

"**Non-Immunogenic, High Molecular Weight Compound**" is a compound of approximately 1000 Da or more that typically does not generate an immunogenic response. For the purposes of this invention, an immunogenic response is one that causes the organism to make antibody proteins. Examples of Non-Immunogenic, High Molecular Weight Compounds include polyethylene glycol (PEG); polysaccharides, such as dextran; polypeptides, such as albumin; and magnetic structures; such as magnetite. In certain embodiments, the Non-Immunogenic, High Molecular Weight Compound can also be a Nucleic Acid Ligand.

"**Lipid Bilayer Vesicles**" are closed, fluid-filled microscopic spheres which are formed principally from individual molecules having polar (hydrophilic) and non-polar (lipophilic) portions. The hydrophilic portions may comprise phosphato, glycerylphosphato, carboxy, sulfato, amino, hydroxy, choline and other polar groups. Examples of non-polar groups are saturated or unsaturated hydrocarbons such as alkyl, alkenyl or other lipid groups. Sterols (e.g., cholesterol) and other pharmaceutically acceptable adjuvants (including anti-oxidants like alpha-tocopherol) may also be included to improve vesicle stability or confer other desirable characteristics.

"**Liposomes**" are a subset of bilayer vesicles and are comprised principally of phospholipid molecules which contain two hydrophobic tails consisting of long fatty acid chains. Upon exposure to water, these molecules spontaneously align to form a bilayer membrane with the lipophilic ends of the molecules in each layer associated in the center of the membrane and the opposing polar ends forming the respective inner and outer surface of the bilayer membrane. Thus, each side of the membrane presents a hydrophilic surface while the interior of the membrane comprises a lipophilic medium. These membranes when formed are generally arranged in a system of concentric closed membranes separated by interlamellar aqueous phases, in a manner not dissimilar to the layers of an onion, around an internal aqueous space. These multilamellar vesicles (MLV) can be converted into small or unilamellar vesicles (UV), with the application of a shearing force.

"**Cationic Liposome**" is a Liposome that contains lipid components that have an overall positive charge at physiological pH.

"**SELEX**" methodology involves the combination of selection of Nucleic Acid Ligands which interact with a Target in a desirable manner, for example binding to a protein, with amplification of those selected Nucleic Acids. Iterative cycling of the selection/amplification steps allows selection of one or a small number of Nucleic Acids

which interact most strongly with the Target from a pool which contains a very large number of Nucleic Acids. Cycling of the selection/amplification procedure is continued until a selected goal is achieved. In the present invention, the SELEX methodology can be employed to obtain a Nucleic Acid Ligand to a desirable Target.

5           The SELEX methodology is described in the SELEX Patent Applications.

**"SELEX Target"** means any compound or molecule of interest for which a ligand is desired. A Target can be a protein (such as VEGF, thrombin, and selectin), peptide, carbohydrate, polysaccharide, glycoprotein, hormone, receptor, antigen, antibody, virus, substrate, metabolite, transition state analog, cofactor, inhibitor, drug, dye, nutrient,  
10   growth factor, etc. without limitation. The terms "SELEX Target" and "Target" can be used interchangeably herein. It will be clear from the sentence context whether or not "Target" means "SELEX Target."

**"Target"** means a preselected location in a biological system including tissues, organs, cells, intracellular compartments, extracellular components. The latter include  
15   hormones (endocrine paracrine, autocrine), enzymes, neurotransmitters and constituents of physiological cascade phenomena (e.g., blood coagulation, complement, etc.).

**"Improved Pharmacokinetic Properties"** means that the Nucleic Acid Ligand in association with the Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound shows a longer circulation half-life *in vivo* relative to the same Nucleic Acid  
20   Ligand not in association with a Lipophilic Compound or Non-Immunogenic, High Molecular Weight Compound or other pharmacokinetic benefits such as improved Target to non-Target concentration ratio.

**"Linker"** is a molecular entity that connects two or more molecular entities through covalent or Non-Covalent Interactions.

25           **"Spacer"** is a Linker of the size that allows spatial separation of two or more molecular entities in a manner that preserves the functional properties of one or more of the molecular entities.

          It is an object of the present invention to provide Complexes comprising one or more Nucleic Acid Ligands in association with a Lipophilic Compound or Non-  
30   Immunogenic, High Molecular Weight Compound. Such Complexes have one or more of the following advantages over a Nucleic Acid Ligand not in association with a Lipophilic Compound or Non-Immunogenic, High Molecular Weight Compound: 1) Improved

Pharmacokinetic Properties, 2) improved capacity for intracellular delivery, or 3) improved capacity for targeting.

The Complexes of the present invention may benefit from one, two, or all three of these advantages. The Complexes of the present invention may contain different Nucleic Acid Ligands serving totally different purposes in the Complex. For example, a Complex of the present invention may be comprised of a) a Liposome, b) a Nucleic Acid Ligand that is targeted to an intracellular SELEX Target that is encapsulated within the interior of the Liposome, and c) a Nucleic Acid Ligand that is targeted to a particular cell type that is associated with and projecting from the exterior surface of the Liposome. In such a case, the Complex will 1) have Improved Pharmacokinetic Properties due to the presence of the Liposome, 2) have enhanced capacity for intracellular delivery of the encapsulated Nucleic Acid Ligand due to the properties of the Liposome, and 3) be specifically targeted to the preselected location *in vivo* by the exteriorly associated Nucleic Acid Ligand.

In another embodiment, the Complex of the present invention is comprised of a Nucleic Acid Ligand covalently attached to a Lipophilic Compound such as cholesterol, dialkyl glycerol, diacyl glycerol, or a Non-Immunogenic, High Molecular Weight Compound such as polyethylene glycol (PEG). In these cases, the pharmacokinetic properties of the Complex will be enhanced relative to the Nucleic Acid Ligand alone. In still other embodiments, the Complex of the present invention is comprised of a Nucleic Acid Ligand encapsulated inside a Liposome, and enhanced intracellular uptake of the Nucleic Acid Ligand is seen over the un-Complexed Nucleic Acid Ligand.

In certain embodiments of the invention, the Complex of the present invention is comprised of a Nucleic Acid Ligand attached to one (dimeric) or more (multimeric) other Nucleic Acid Ligands. The Nucleic Acid Ligand can be to the same or different SELEX Target. In embodiments where there are multiple Nucleic Acid Ligands to the same SELEX Target, there is an increase in avidity due to multiple binding interactions with the SELEX Target. Furthermore, in embodiments of the invention where the Complex is comprised of a Nucleic Acid Ligand attached to one or more other Nucleic Acid Ligands, the pharmacokinetic properties of the Complex will be improved relative to one Nucleic Acid Ligand alone.

The Lipophilic Compound or Non-Immunogenic, High Molecular Weight Compound can be covalently bonded or associated through Non-Covalent Interactions with the Nucleic Acid Ligand(s). In embodiments where the Lipophilic Compound is

cholesterol, dialkyl glycerol, diacyl glycerol, or the Non-Immunogenic, High Molecular Weight Compound is PEG, a covalent association with the Nucleic Acid Ligand(s) is preferred. In embodiments where the Lipophilic Compound is a Cationic Liposome or where the Nucleic Acid Ligands are encapsulated within the Liposome, a non-covalent association with the Nucleic Acid Ligand(s) is preferred. In embodiments where covalent attachment is employed, the Lipophilic Compound or Non-Immunogenic, High Molecular Weight Compound may be covalently bound to a variety of positions on the Nucleic Acid Ligand, such as to an exocyclic amino group on the base, the 5-position of a pyrimidine nucleotide, the 8-position of a purine nucleotide, the hydroxyl group of the phosphate, or a hydroxyl group or other group at the 5' or 3' terminus of the Nucleic Acid Ligand. Preferably, however, it is bonded to the 5' or 3' hydroxyl group thereof. Attachment of the Nucleic Acid Ligand to other components of the Complex can be done directly or with the utilization of Linkers or Spacers.

The Lipophilic Compound or Non-Immunogenic, High Molecular Weight Compound can associate through Non-Covalent Interactions with the Nucleic Acid Ligand(s). For example, in one embodiment of the present invention, the Nucleic Acid Ligand is encapsulated within the internal compartment of the Lipophilic Compound. In another embodiment of the present invention, the Nucleic Acid Ligand associates with the Lipophilic Compound through electrostatic interactions. For instance, a Cationic Liposome can associate with an anionic Nucleic Acid Ligand. Another example of a Non-Covalent Interaction through ionic attractive forces is one in which a portion of the Nucleic Acid Ligand hybridizes through Watson-Crick base-pairing or triple helix base pairing to an oligonucleotide which is associated with a Lipophilic Compound or Non-Immunogenic, High Molecular Weight Compound.

One problem encountered in the therapeutic and *in vivo* diagnostic use of Nucleic Acids is that oligonucleotides in their phosphodiester form may be quickly degraded in body fluids by intracellular and extracellular enzymes such as endonucleases and exonucleases before the desired effect is manifest. Certain chemical modifications of the Nucleic Acid Ligand can be made to increase the *in vivo* stability of the Nucleic Acid Ligand or to enhance or to mediate the delivery of the Nucleic Acid Ligand. Modifications of the Nucleic Acid Ligands contemplated in this invention include, but are not limited to, those which provide other chemical groups that incorporate additional charge, polarizability, hydrophobicity, hydrogen bonding, electrostatic interaction, and

fluxionality to the Nucleic Acid Ligand bases or to the Nucleic Acid Ligand as a whole. Such modifications include, but are not limited to, 2'-position sugar modifications, 5-position pyrimidine modifications, 8-position purine modifications, modifications at exocyclic amines, substitution of 4-thiouridine, substitution of 5-bromo or 5-iodo-uracil; backbone modifications, phosphorothioate or alkyl phosphate modifications, methylations, unusual base-pairing combinations such as the isobases isocytidine and isoguanidine and the like. Modifications can also include 3' and 5' modifications such as capping.

Where the Nucleic Acid Ligands are derived by the SELEX method, the modifications can be pre- or post- SELEX modifications. Pre-SELEX modifications yield Nucleic Acid Ligands with both specificity for their SELEX Target and improved *in vivo* stability. Post-SELEX modifications made to 2'-OH Nucleic Acid Ligands can result in improved *in vivo* stability without adversely affecting the binding capacity of the Nucleic Acid Ligands. The preferred modifications of the Nucleic Acid Ligands of the subject invention are 5' and 3' phosphorothioate capping or 3'3' inverted phosphodiester linkage at the 3' end. For RNA ligands, additional 2' amino (2'-NH<sub>2</sub>) modification of some or all of the nucleotides is preferred.

In another aspect of the present invention, the association of the Nucleic Acid Ligand with a Lipophilic Compound or Non-Immunogenic, High Molecular Weight Compound, results in Improved Pharmacokinetic Properties (i.e., slower clearance rate) relative to the Nucleic Acid Ligand not in association with a Lipophilic Compound or Non-Immunogenic, High Molecular Weight Compound. In one embodiment of the invention, the Complex includes a Lipid Construct. The Complex with the Nucleic Acid Ligand can be formed through covalent or Non-Covalent Interactions. In a preferred embodiment, the Lipid Construct is a Lipid Bilayer Vesicle. In the most preferred embodiment, the Lipid Construct is a Liposome.

In certain embodiments of this invention, the Complex comprises a Liposome with a targeting Nucleic Acid Ligand projecting out of the Liposome. In embodiments where there are multiple Nucleic Acid Ligands to the same Target, there is an increase in avidity due to multiple binding interactions with the Target.

Liposomes for use in the present invention can be prepared by any of the various techniques presently known in the art or subsequently developed. Typically, they are prepared from a phospholipid, for example, distearoyl phosphatidylcholine, and may

include other materials such as neutral lipids, for example, cholesterol, and also surface modifiers such as positively charged (e.g., sterylamine or aminomannose or aminomannitol derivatives of cholesterol) or negatively charged (e.g., dicetyl phosphate, phosphatidyl glycerol) compounds. Multilamellar Liposomes can be formed by the conventional  
5 technique, that is, by depositing a selected lipid on the inside wall of a suitable container or vessel by dissolving the lipid in an appropriate solvent, and then evaporating the solvent to leave a thin film on the inside of the vessel or by spray drying. An aqueous phase is then added to the vessel with a swirling or vortexing motion which results in the formation of MLVs. UVs can then be formed by homogenization, sonication or extrusion (through  
10 filters) of MLV's. In addition, UVs can be formed by detergent removal techniques.

In certain embodiments of this invention, the Complex comprises a Liposome with a targeting Nucleic Acid Ligand(s) associated with the surface of the Liposome and an encapsulated therapeutic or diagnostic agent. Preformed Liposomes can be modified to associate with the Nucleic Acid Ligands. For example, a Cationic Liposome associates  
15 through electrostatic interactions with the Nucleic Acid Ligand. Alternatively, a Nucleic Acid Ligand attached to a Lipophilic Compound, such as cholesterol, can be added to preformed Liposomes whereby the cholesterol becomes associated with the liposomal membrane. Alternatively, the Nucleic Acid Ligand can be associated with the Liposome during the formulation of the Liposome. Preferably, the Nucleic Acid Ligand is associated  
20 with the Liposome by loading into preformed Liposomes.

It is well known in the art that Liposomes are advantageous for encapsulating or incorporating a wide variety of therapeutic and diagnostic agents. Any variety of compounds can be enclosed in the internal aqueous compartment of the Liposomes. Illustrative therapeutic agents include antibiotics, antiviral nucleosides, antifungal  
25 nucleosides, metabolic regulators, immune modulators, chemotherapeutic drugs, toxin antidotes, DNA, RNA, antisense oligonucleotides, etc. By the same token, the Lipid Bilayer Vesicles may be loaded with a diagnostic radionuclide (e.g., Indium 111, Iodine 131, Yttrium 90, Phosphorous 32, or gadolinium) and fluorescent materials or other materials that are detectable in *in vitro* and *in vivo* applications. It is to be understood that  
30 the therapeutic or diagnostic agent can be encapsulated by the Liposome walls in the aqueous interior. Alternatively, the carried agent can be a part of, that is, dispersed or dissolved in the vesicle wall-forming materials.

During Liposome formation, water soluble carrier agents may be encapsulated in the aqueous interior by including them in the hydrating solution, and lipophilic molecules incorporated into the lipid bilayer by inclusion in the lipid formulation. In the case of certain molecules (e.g., cationic or anionic lipophilic drugs), loading of the drug into preformed Liposomes may be accomplished, for example, by the methods described in U.S. Patent No. 4,946,683, the disclosure of which is incorporated herein by reference. Following drug encapsulation, the Liposomes are processed to remove unencapsulated drug through processes such as gel chromatography or ultrafiltration. The Liposomes are then typically sterile filtered to remove any microorganisms which may be present in the suspension. Microorganisms may also be removed through aseptic processing.

If one wishes to encapsulate large hydrophilic molecules with Liposomes, larger unilamellar vesicles can be formed by methods such as the reverse-phase evaporation (REV) or solvent infusion methods. Other standard methods for the formation of Liposomes are known in the art, for example, methods for the commercial production of Liposomes include the homogenization procedure described in U.S. Patent No. 4,753,788 and the thin-film evaporation method described in U.S. Patent No. 4,935,171, which are incorporated herein by reference.

It is to be understood that the therapeutic or diagnostic agent can also be associated with the surface of the Lipid Bilayer Vesicle. For example, a drug can be attached to a phospholipid or glyceride (a prodrug). The phospholipid or glyceride portion of the prodrug can be incorporated into the lipid bilayer of the Liposome by inclusion in the lipid formulation or loading into preformed Liposomes (see U.S. Patent Nos 5,194,654 and 5,223,263, which are incorporated by reference herein).

It is readily apparent to one skilled in the art that the particular Liposome preparation method will depend on the intended use and the type of lipids used to form the bilayer membrane.

A Nucleic Acid Ligand or ligands in association with a Lipophilic Compound or Non-Immunogenic, High Molecular Weight Compound may enhance the intracellular delivery of the Nucleic Acid Ligand(s) over non-associated Nucleic Acid Ligand(s). The efficiency of delivery of the Complex to cells may be optimized by using lipid formulations and conditions known to enhance fusion of Liposomes with cellular membranes. For example, certain negatively charged lipids such as phosphatidylglycerol and phosphatidylserine promote fusion, especially in the presence of other fusogens (e.g.,



multivalent cations like  $\text{Ca}^{2+}$ , free fatty acids, viral fusion proteins, short chain PEG, lysolecithin, detergents and surfactants). Phosphatidylethanolamine may also be included in the Liposome formulation to increase membrane fusion and, concomitantly, enhance cellular delivery. In addition, free fatty acids and derivatives thereof, containing, for

5 example, carboxylate moieties, may be used to prepare pH-sensitive Liposomes which are negatively charged at higher pH and neutral or protonated at lower pH. Such pH-sensitive Liposomes are known to possess a greater tendency to fuse.

In the preferred embodiment, the Nucleic Acid Ligands of the present invention are derived from the SELEX methodology. SELEX is described in U.S. Patent Application

10 Serial No. 07/536,428, entitled Systematic Evolution of Ligands by EXponential Enrichment, now abandoned, U.S. Patent Application Serial No. 07/714,131, filed June 10, 1991, entitled Nucleic Acid Ligands, now United States Patent No. 5,475,096, United States Patent Application Serial No. 07/931,473, filed August 17, 1992, entitled Nucleic Acid Ligands, now United States Patent No. 5,270,163 (see also PCT/US91/04078).

15 These applications, each specifically incorporated herein by reference, are collectively called the SELEX Patent Applications.

The SELEX process provides a class of products which are Nucleic Acid molecules, each having a unique sequence, and each of which has the property of binding specifically to a desired Target compound or molecule. Target molecules are preferably

20 proteins, but can also include among others carbohydrates, peptidoglycans and a variety of small molecules. SELEX methodology can also be used to Target biological structures, such as cell surfaces or viruses, through specific interaction with a molecule that is an integral part of that biological structure.

In its most basic form, the SELEX process may be defined by the following series

25 of steps:

1) A Candidate Mixture of Nucleic Acids of differing sequence is prepared. The Candidate Mixture generally includes regions of fixed sequences (i.e., each of the members of the Candidate Mixture contains the same sequences in the same location) and regions of randomized sequences. The fixed sequence regions are selected either: (a) to assist in the

30 amplification steps described below, (b) to mimic a sequence known to bind to the Target, or (c) to enhance the concentration of a given structural arrangement of the Nucleic Acids in the Candidate Mixture. The randomized sequences can be totally randomized (i.e., the probability of finding a base at any position being one in four) or only partially

randomized (e.g., the probability of finding a base at any location can be selected at any level between 0 and 100 percent).

2) The Candidate Mixture is contacted with the selected Target under conditions favorable for binding between the Target and members of the Candidate Mixture. Under these circumstances, the interaction between the Target and the Nucleic Acids of the Candidate Mixture can be considered as forming Nucleic Acid-target pairs between the Target and those Nucleic Acids having the strongest affinity for the Target.

3) The Nucleic Acids with the highest affinity for the target are partitioned from those Nucleic Acids with lesser affinity to the target. Because only an extremely small number of sequences (and possibly only one molecule of Nucleic Acid) corresponding to the highest affinity Nucleic Acids exist in the Candidate Mixture, it is generally desirable to set the partitioning criteria so that a significant amount of the Nucleic Acids in the Candidate Mixture (approximately 5-50%) are retained during partitioning.

4) Those Nucleic Acids selected during partitioning as having the relatively higher affinity for the target are then amplified to create a new Candidate Mixture that is enriched in Nucleic Acids having a relatively higher affinity for the target.

5) By repeating the partitioning and amplifying steps above, the newly formed Candidate Mixture contains fewer and fewer unique sequences, and the average degree of affinity of the Nucleic Acids to the target will generally increase. Taken to its extreme, the SELEX process will yield a Candidate Mixture containing one or a small number of unique Nucleic Acids representing those Nucleic Acids from the original Candidate Mixture having the highest affinity to the target molecule.

The basic SELEX method has been modified to achieve a number of specific objectives. For example, United States Patent Application Serial No. 07/960,093, filed October 14, 1992, entitled "Method for Selecting Nucleic Acids on the Basis of Structure", describes the use of SELEX in conjunction with gel electrophoresis to select Nucleic Acid molecules with specific structural characteristics, such as bent DNA. United States Patent Application Serial No. 08/123,935, filed September 17, 1993, entitled "Photoselection of Nucleic Acid Ligands" describes a SELEX based method for selecting Nucleic Acid Ligands containing photoreactive groups capable of binding and/or photocrosslinking to and/or photoinactivating a target molecule. United States Patent Application Serial No. 08/134,028, filed October 7, 1993, entitled "High-Affinity Nucleic Acid Ligands That Discriminate Between Theophylline and Caffeine", describes a method for identifying

highly specific Nucleic Acid Ligands able to discriminate between closely related molecules, termed Counter-SELEX. United States Patent Application Serial No. 08/143,564, filed October 25, 1993, entitled "Systematic Evolution of Ligands by EXponential Enrichment: Solution SELEX", describes a SELEX-based method which  
5 achieves highly efficient partitioning between oligonucleotides having high and low affinity for a target molecule. United States Patent Application Serial No. 07/964,624, filed October 21, 1992, entitled "Methods of Producing Nucleic Acid Ligands" describes methods for obtaining improved Nucleic Acid Ligands after SELEX has been performed. United States Patent Application Serial No. 08/400,440, filed March 8, 1995, entitled  
10 "Systematic Evolution of Ligands by EXponential Enrichment: Chemi-SELEX", describes methods for covalently linking a ligand to its target.

The SELEX method encompasses the identification of high-affinity Nucleic Acid Ligands containing modified nucleotides conferring improved characteristics on the ligand, such as improved *in vivo* stability or improved delivery characteristics. Examples of such  
15 modifications include chemical substitutions at the ribose and/or phosphate and/or base positions. SELEX-identified Nucleic Acid Ligands containing modified nucleotides are described in United States Patent Application Serial No. 08/117,991, filed September 8, 1993, entitled "High Affinity Nucleic Acid Ligands Containing Modified Nucleotides", that describes oligonucleotides containing nucleotide derivatives chemically modified at the  
20 5- and 2'-positions of pyrimidines. United States Patent Application Serial No. 08/134,028, *supra*, describes highly specific Nucleic Acid Ligands containing one or more nucleotides modified with 2'-amino (2'-NH<sub>2</sub>), 2'-fluoro (2'-F), and/or 2'-O-methyl (2'-OMe). United States Patent Application Serial No. 08/264,029, filed June 22, 1994, entitled "Novel Method of Preparation of 2' Modified Pyrimidine Intramolecular  
25 Nucleophilic Displacement", describes oligonucleotides containing various 2'-modified pyrimidines.

The SELEX method encompasses combining selected oligonucleotides with other selected oligonucleotides and non-oligonucleotide functional units as described in United States Patent Application Serial No. 08/284,063, filed August 2, 1994, entitled  
30 "Systematic Evolution of Ligands by Exponential Enrichment: Chimeric SELEX" and United States Patent Application Serial No. 08/234,997, filed April 28, 1994, entitled "Systematic Evolution of Ligands by Exponential Enrichment: Blended SELEX", respectively. These applications allow the combination of the broad array of shapes and

other properties, and the efficient amplification and replication properties, of oligonucleotides with the desirable properties of other molecules. Each of the above described patent applications which describe modifications of the basic SELEX procedure are specifically incorporated by reference herein in their entirety.

5 SELEX identifies Nucleic Acid Ligands that are able to bind targets with high affinity and with outstanding specificity, which represents a singular achievement that is unprecedented in the field of Nucleic Acids research. These characteristics are, of course, the desired properties one skilled in the art would seek in a therapeutic or diagnostic ligand.

10 In order to produce Nucleic Acid Ligands desirable for use as a pharmaceutical, it is preferred that the Nucleic Acid Ligand (1) binds to the target in a manner capable of achieving the desired effect on the target; (2) be as small as possible to obtain the desired effect; (3) be as stable as possible; and (4) be a specific ligand to the chosen target. In most situations, it is preferred that the Nucleic Acid Ligand has the highest possible affinity to the target. Additionally, Nucleic Acid Ligands can have facilitating properties.

15 In co-pending and commonly assigned U.S. Patent Application Serial No. 07/964,624, filed October 21, 1992 ('624), methods are described for obtaining improved Nucleic Acid Ligands after SELEX has been performed. The '624 application, entitled Methods of Producing Nucleic Acid Ligands, is specifically incorporated herein by reference.

20 In embodiments where the Nucleic Acid Ligand(s) can serve in a targeting capacity, the Nucleic Acid Ligands adopt a three dimensional structure that must be retained in order for the Nucleic Acid Ligand to be able to bind its target. In addition, the Nucleic Acid Ligand must be properly oriented with respect to the surface of the Complex so that its Target binding capacity is not compromised. This can be accomplished by  
25 attaching the Nucleic Acid Ligand at a position that is distant from the binding portion of the Nucleic Acid Ligand. The three dimensional structure and proper orientation can also be preserved by use of a Linker or Spacer as described *supra*.

Any variety of therapeutic or diagnostic agents can be attached, encapsulated, or incorporated into the Complex as discussed *supra* for targeted delivery by the Complex.

30 In embodiments where the Complex is comprised of a Liposome and a Nucleic Acid Ligand, for example, a fungi-specific Nucleic Acid Ligand exposed on the surface of the Complex could Target a fungal cell for delivery of a fungicide (e.g., amphotericin B).

Alternatively, a chemotherapeutic agent can be delivered to tumor cells via a Nucleic Acid Ligand to a tumor antigen.

In an alternative embodiment, the therapeutic or diagnostic agent to be delivered to the Target cell could be another Nucleic Acid Ligand. For example, a Nucleic Acid Ligand  
5 that binds to a tumor antigen could be presented to the outside of the Complex, and a Nucleic Acid Ligand that binds to and inhibits the mutated isoform of an intracellular Target such as p21, the protein product of the ras gene, could be the agent to be delivered.

It is further contemplated by this invention that the agent to be delivered can be incorporated into the Complex in such a way as to be associated with the outside surface  
10 of the Complex. (e.g., a prodrug, receptor antagonist, or radioactive substance for treatment or imaging). As with the Nucleic Acid Ligand, the agent can be associated through covalent or Non-Covalent Interactions. The Complex would provide targeted delivery of the agent extracellularly, with the Liposome serving as a Linker.

In another embodiment, a Non-Immunogenic, High Molecular Weight Compound  
15 (e.g., PEG) can be attached to the Liposome to provide Improved Pharmacokinetic Properties for the Complex. Nucleic Acid Ligands may be attached to the Liposome membrane as described supra or may be attached to a Non-Immunogenic, High Molecular Weight Compound which in turn is attached to the membrane. In this way, the Complex may be shielded from blood proteins and thus be made to circulate for extended periods of  
20 time while the Nucleic Acid Ligand is still sufficiently exposed to make contact with and bind to its SELEX Target.

In one embodiment of this invention, the Nucleic Acid Ligand presented on the outside of the Complex can Target circulating proteins (e.g., antibodies, growth factors, protein hormones) for removal by the reticuloendothelial system (i.e., liver and spleen). As  
25 an example, the treatment of autoimmune diseases may be possible by such a Complex. Autoimmune diseases are the result of a failure of an organism's immune system to avoid recognition of self due to production of autoantibodies and autoreactive T cells. The attack by the immune system on host cells can result in a large number of disorders including neural diseases, such as multiple sclerosis and myasthenia gravis; diseases of the joints,  
30 such as rheumatoid arthritis; attacks on Nucleic Acids, as observed with systemic lupus erythematosus; and such other diseases associated with various organs, as psoriasis, juvenile onset diabetes, Sjogren's disease, and Graves disease. As it has been found that Liposomes associated with proteins are generally cleared by the reticuloendothelial system

(i.e., spleen and liver) faster than Liposomes without associated proteins, Nucleic Acid Ligands complexed with a Liposome, can be used for removal of autoantibodies by the reticuloendothelial system.

In another embodiment of the present invention, Nucleic Acid Ligands specific for  
5 the same SELEX Target are attached to the surface of the same Liposome. This provides the possibility of bringing the same SELEX Targets in close proximity to each other and can be used to generate specific interactions between the same SELEX Targets. For example, Nucleic Acid Ligands to a tyrosine kinase receptor attached to a Liposome would bring the receptors in close proximity to one another. This would facilitate  
10 autophosphorylation which would initiate a signal transduction cascade.

In an alternative embodiment of the present invention, Nucleic Acid Ligands specific for different SELEX Targets are attached to the surface of the same Liposome. This provides the possibility of bringing the distinct Targets in close proximity to each other and can be used to generate specific interactions between the Targets. For example,  
15 Nucleic Acid Ligands specific for a tumor marker or antigen and Nucleic Acid Ligands specific for a T-cell receptor would bring the T-cells in close proximity to the tumor. In addition to using the Liposome as a way of bringing Targets in close proximity, agents could be encapsulated in the Liposome (e.g., immune system modulator) to increase the intensity of the interaction (e.g., increase the T-cell immune response).

20 In instances where it is difficult to identify biomolecules that are unique to a cellular Target of interest, specificity may be obtained by having Nucleic Acid Ligands that are specific for two or more markers to the Target associated with the Complex. In this scenario, it is expected that the best Nucleic Acid Ligands would have low or medium affinity for their respective Targets. The use of Nucleic Acid Ligands of this type are  
25 recommended since high affinity Nucleic Acid Ligands would lead to the association of drug with all cells possessing either marker protein, thereby reducing specificity. With lower affinity ligands, avidity is required to provide the necessary specificity.

The Liposome/Nucleic Acid Ligand Complex also allows for the possibility of multiple binding interactions to the Target. This, of course, depends on the number of  
30 Nucleic Acid Ligands per Complex and mobility of the Nucleic Acid Ligands and receptors in their respective membranes. Since the effective binding constant may increase as the product of the binding constant for each site, there is a substantial advantage to having multiple binding interactions. In other words, by having many Nucleic Acid Ligands

attached to the Liposome, and therefore creating multivalency, the effective affinity (i.e., the avidity) of the multimeric Complex for its Target may become as good as the product of the binding constant for each site.

In certain embodiments of the invention, the Complex of the present invention is comprised of a Nucleic Acid Ligand attached to a Lipophilic Compound such as cholesterol, or dialkyl glycerol, or diacyl glycerol. In this case, the pharmacokinetic properties of the Complex will be improved relative to the Nucleic Acid Ligand alone. As discussed *supra*, cholesterol may be covalently bound to the Nucleic Acid Ligand at numerous positions on the Nucleic Acid Ligand. In another embodiment of the invention, the Complex may further comprise a Lipid Construct such as a Liposome. In this embodiment, the cholesterol can assist in the incorporation of the Nucleic Acid Ligand into the Liposome due to the propensity for cholesterol to associate with other Lipophilic Compounds. The cholesterol in association with a Nucleic Acid Ligand can be incorporated into the lipid bilayer of the Liposome by inclusion in the formulation or by loading into preformed Liposomes. In the preferred embodiment, the cholesterol/Nucleic Acid Ligand Complex is associated with a preformed Liposome. The cholesterol can associate with the membrane of the Liposome in such a way so as the Nucleic Acid Ligand is projecting into or out of the Liposome. In embodiments where the Nucleic Acid Ligand is projecting out of the Complex, the Nucleic Acid Ligand can serve in a targeting capacity.

In other embodiments, the Complex of the present invention is comprised of a Nucleic Acid Ligand attached to a Non-Immunogenic, High Molecular Weight Compound such as PEG, dialkyl glycerol, diacyl glycerol, or cholesterol. In this embodiment, the pharmacokinetic properties of the Complex are improved relative to the Nucleic Acid Ligand alone. As discussed *supra*, the association could be through Covalent Bonds or Non-Covalent Interactions. In the preferred embodiment, the Nucleic Acid Ligand is associated with the PEG, dialkyl glycerol, diacyl glycerol, or a cholesterol molecule through Covalent Bonds. Also, as discussed *supra*, where covalent attachment is employed, PEG, dialkyl glycerol, diacyl glycerol, or cholesterol may be covalently bound to a variety of positions on the Nucleic Acid Ligand. In embodiments where PEG or diacyl glycerol are used, it is preferred that the Nucleic Acid Ligand is bonded to the 5' thiol through a maleimide or vinyl sulfone functionality or via a phosphodiester linkage. In embodiments where dialkyl glycerol and cholesterol are used, it is preferred that the Nucleic Acid Ligand is bonded via a phosphodiester linkage. In certain embodiments, a

plurality of Nucleic Acid Ligands can be associated with a single PEG, dialkyl glycerol, diacyl glycerol, or cholesterol molecule. The Nucleic Acid Ligands can be to the same or different Target. In embodiments where there are multiple Nucleic Acid Ligands to the same Target, there is an increase in avidity due to multiple binding interactions with the Target. In yet further embodiments, a plurality of PEG, dialkyl glycerol, diacyl glycerol, or cholesterol molecules can be attached to each other. In these embodiments, one or more Nucleic Acid Ligands to the same Target or different Targets can be associated with each PEG, dialkyl glycerol, diacyl glycerol, or cholesterol molecule. This also results in an increase in avidity of each Nucleic Acid Ligand to its SELEX Target. In embodiments where multiple Nucleic Acids specific for the same SELEX Target are attached to PEG, dialkyl glycerol, diacyl glycerol, or cholesterol, there is the possibility of bringing the same Targets in close proximity to each other in order to generate specific interactions between the same Targets. Where multiple Nucleic Acid Ligands specific for different Targets are attached to PEG, dialkyl glycerol, diacyl glycerol, or cholesterol, there is the possibility of bringing the distinct Targets in close proximity to each other in order to generate specific interactions between the Targets. In addition, in embodiments where there are Nucleic Acid Ligands to the same Target or different Targets associated with PEG, dialkyl glycerol, diacyl glycerol, or cholesterol, a drug can also be associated with PEG, dialkyl glycerol, diacyl glycerol, or cholesterol. Thus the Complex would provide targeted delivery of the drug, with PEG, dialkyl glycerol, diacyl glycerol, or cholesterol serving as a Linker.

In another embodiment of the invention, the Complex is comprised of a Nucleic Acid Ligand attached to a Non-Immunogenic, High Molecular Weight Compound such as magnetite. As discussed supra, the association could be through Covalent Bonds or Non-Covalent Interactions. In the preferred embodiment, the Nucleic Acid Ligand is associated with magnetite through Covalent Bonds. The magnetite can be coated with a variety of compounds that display different functional chemistries for attachment (e.g., dextran, Lipophilic Compounds). The Nucleic Acid Ligand in association with the magnetite provides targeted delivery of the magnetite for use in nuclear magnetic resonance imaging.

The following examples are provided to explain and illustrate the present invention and are not to be taken as limiting of the invention. The structures of the Nucleic Acid Ligands described in the examples below are shown in Figure 1. Example 1 describes the conjugation of Nucleic Acid Ligands with lipid, dialkyl glycerol or diacyl glycerol, as well as incorporation of pharmacokinetic modifiers via automated synthesis. Example 2



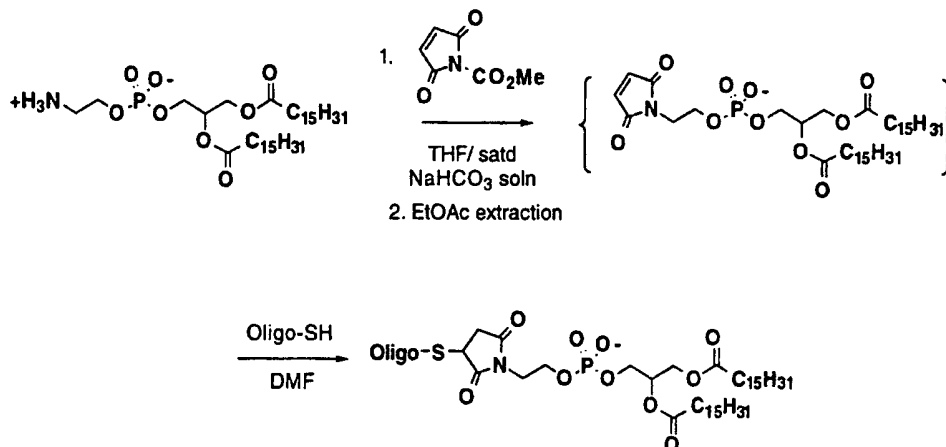
describes the conjugation of PEG and cholesterol with a Nucleic Acid Ligand. The modifications to the Nucleic Acid Ligand do not interfere with its ability to bind to its SELEX Target, as the binding affinities of the PEG-conjugated and cholesterylated Nucleic Acid Ligands were identical to the non-conjugated and non-cholesterylated molecules.

- 5 Example 3 describes the incorporation of a cholesterol-derivatized Nucleic Acid Ligand into a lipid formulation. The activity of the Nucleic Acid Ligand/Liposome formulations containing thrombin Nucleic Acid Ligands was tested in an *in vitro* clotting inhibition assay. Liposome processing conditions do not affect the anticoagulation activity of the Nucleic Acid Ligand. In addition, the liposomal association does not affect the ability of
- 10 the Nucleic Acid Ligand to bind and inhibit its Target. Example 4 describes the pharmacokinetic properties of Nucleic Acid Ligands in association with cholesterol alone, dialkyl glycerol alone, with PEG alone, with cholesterol and Liposome, with dialkyl glycerol and Liposomes, and with PEG and Liposome. Nucleic Acid Ligands that have been modified at the 2' sugar position of purines and pyrimidines are also included.
- 15 Example 5 reports on the toxicity and intracellular uptake by human lymphocytes of Cationic Liposome-Nucleic Acid Ligand Complexes. Examples 6 - 10 describe the following effects on the incorporation of Nucleic Acid Ligands into preformed Liposomes: varying the negative charge of the lipids, varying the cholesterol content, varying the lipid/Nucleic Acid Ligand ratio with a fixed amount of Nucleic Acid Ligand, varying the
- 20 lipid/Nucleic Acid Ligand ratio with a fixed amount of SUV, and varying the phospholipid chain length. Example 11 demonstrates that incorporation of a Nucleic Acid Ligand/cholesterol conjugate into a liposomal formulation has occurred via non-denaturing gel electrophoresis. Example 12 describes the way in which Nucleic Acid Ligands can be passively encapsulated into Liposomes. Example 13 describes the way in which Nucleic
- 25 Acid Ligands can be remotely loaded into Liposomes. Example 14 describes the covalent conjugation of Nucleic Acid Ligands to Liposomes. Example 15 describes the *in vitro* and *in vivo* efficacy of a Nucleic Acid Ligand-Liposome Complex.

**EXAMPLE 1. LIPID, PEG, DIALKYL GLYCEROL AND DIACYL GLYCEROL**  
**REAGENTS FOR OLIGONUCLEOTIDE MODIFICATION**

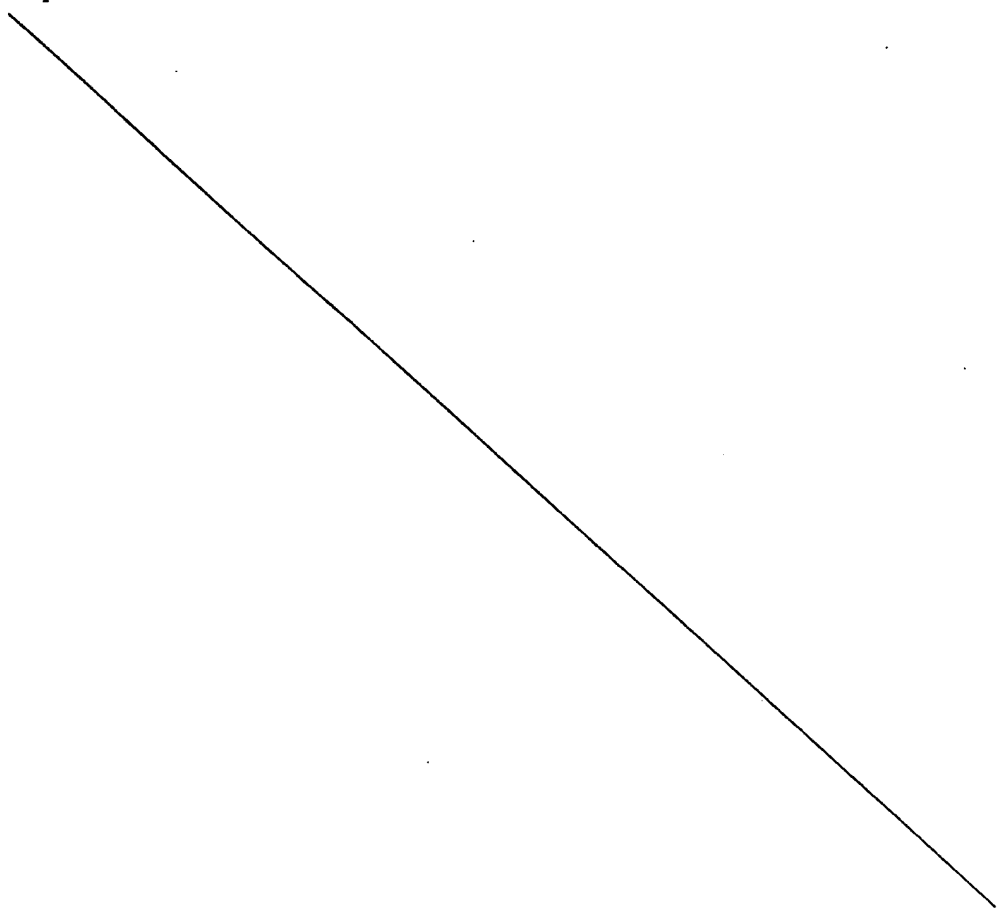
30 In this example, conjugation of Nucleic Acid Ligands with lipid and/or PEG or diacyl glycerol or dialkyl glycerol reagents is described, as well as incorporation of the pharmacokinetic modifiers via automated synthesis using either phosphoramidite or H-

phosphonate coupling chemistry. In the schemes depicted below, a solid arrow represents steps that have been completed, whereas a dashed arrow represents steps that have not yet been completed. **Scheme 1** depicts the preparation of a dipalmitoyl phosphatidyl ethanolamine maleimide reagent for coupling to sulfhydryl-modified oligonucleotide substrates. This procedure is analogous to that reported by Cheronis *et al* (Cheronis, J. C. *et al.*, *J. Med. Chem.* (1992) 35:1563-1572) for the preparation of bis- and tris-maleimide reagents from simple, aliphatic di- and triamine substrates. Treatment of the phospholipid with methoxycarbonyl maleimide resulted in formation of an uncharacterized intermediate which, upon incubation with sulfhydryl-modified oligonucleotide, resulted in complete conversion of the oligonucleotide to the dipalmitoyl phosphatidyl ethanolamine conjugate (*vide infra*). Similar reagents are also available commercially from Avanti Polar Lipids.

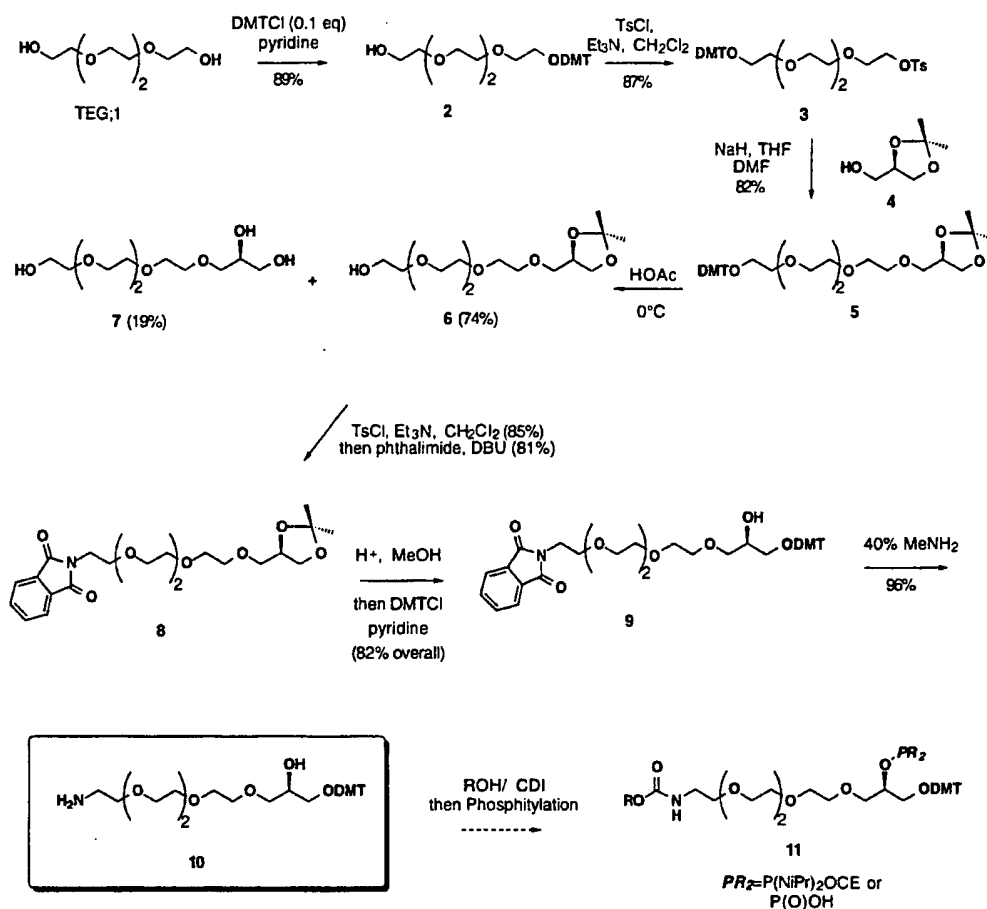
**Scheme 1**

The ability to modify oligonucleotides under automated synthesis conditions has many obvious advantages. Reagents were prepared which allowed facile incorporation of lipid and/or PEG moieties under standard automated synthesis conditions. Initially, the versatile module **10** was designed and synthesized (**Scheme 2**) which could be divergently converted to any number of oligo modification reagents of interest by simply chemoselectively functionalizing the amine group then phosphitylation of the hydroxyl group. Noteworthy is the flexibility this strategy offers with respect to the modification group (the amine ligand) and the activated phosphate precursor (phosphoramidite, H-phosphonate, or phosphate triester) introduced via derivatization of the 2'-hydroxyl group. Additionally, the glycerol nucleus of automated synthesis reagents prepared in this

way renders the products suitable for oligo modification at internal chain positions, or at the 5' end. An alternative synthesis of module 10 is shown in Scheme 3. Tetraethylene glycol (1; TEG) is derivatized as the monotosylate 2a upon treatment with a limiting amount of *p*-toluenesulfonyl chloride, preferably 10 mole percent, in a basic medium, preferably pyridine. In this manner, 2a was obtained in 75% yield after silica gel filtration. Conversion of 2a to the TEG phthalimide 3a was accomplished in 80% yield upon treatment with phthalimide in the presence of diazabicycloundecane (DBU) as a base at elevated temperature in DMF solution. Allylation of phthalimide 3a (allyl bromide, NaH, THF/ DMF) afforded 65% yield of allyl TEG 4a. Treatment of 4a with 0.5% OsO<sub>4</sub> and 1.1 equivalents of N-methylmorpholine N-oxide (NMO) afforded a diol intermediate that was, without further purification, converted to the dimethoxytrityl (DMT) ether derivative 9 in 89% overall yield for the two steps. Finally, amine deprotection using 40% MeNH<sub>2</sub> was carried out to afford 10 in 95% purified yield. Module 10 has been further elaborated by treatment with PEG-nitrophenylcarbonate (PEG-NPC; Shearwater Polymers). In this way, the phosphitylation precursor 12 (Scheme 4) was prepared in excellent yield. Further conversions of 12 to both phosphoramidite 13 and H-phosphonate 14 have been carried out.

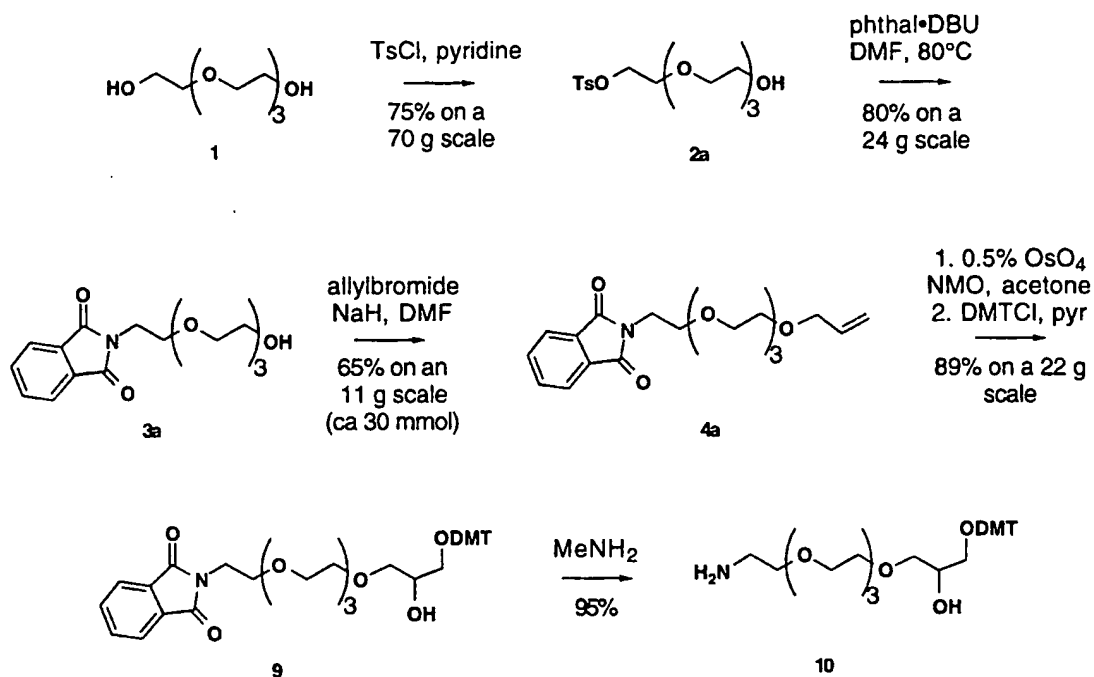


### Scheme 2



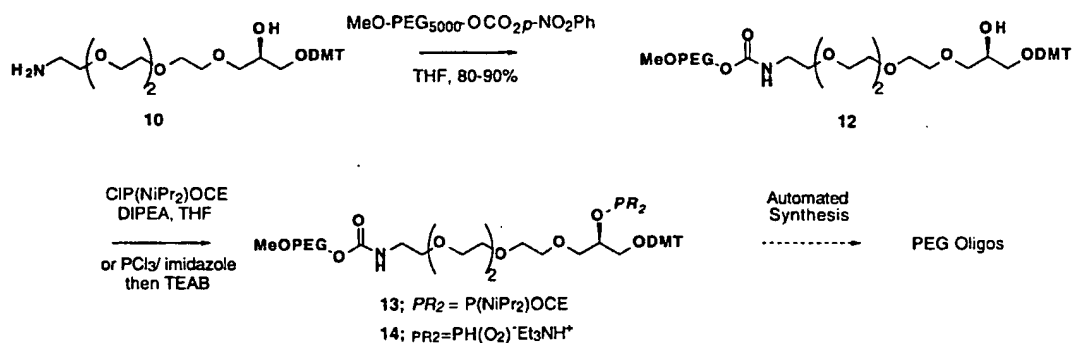
**Phosphoramidites/ H-Phosphonates; ROH=PEG, lipids, metal chelators, hydroxyphenyls (for radioiodination), etc...**

Scheme 3



5

Scheme 4

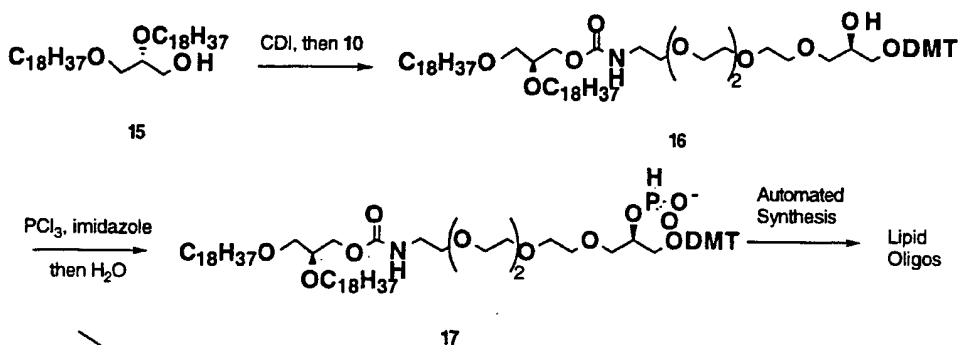


10

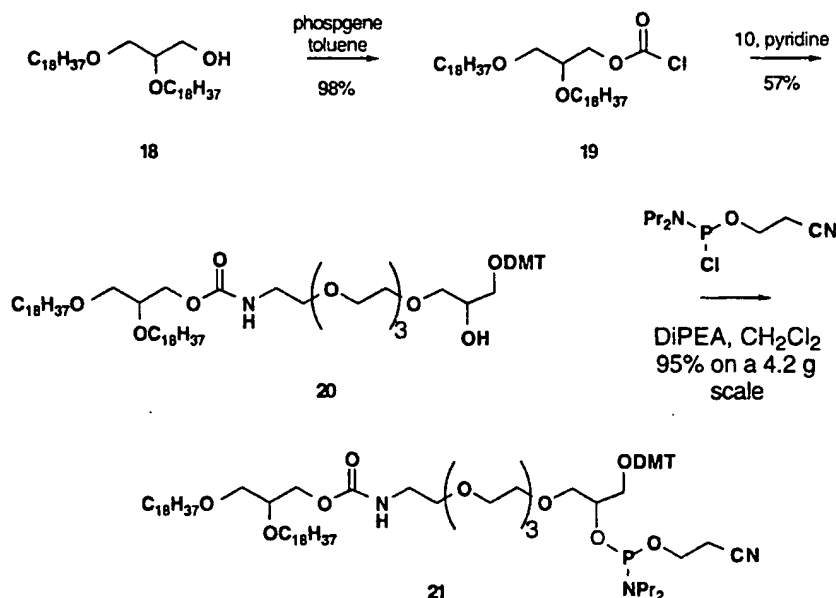
The design of a lipid reagent for oligonucleotide modification by automated synthesis necessitates replacement of the ester linkages of native diacyl glycerols, as in the dipalmitoyl phosphatidyl derivative described above, by glycerol-alkyl linkages stable to the basic deprotection protocol required for synthetic oligo recovery. The linkage that was chosen to explore initially was the ether linkage, as in the known dipalmityl glycerol derivative **15** (available from Sigma), although long-chain alkyl carbamates (or a combination of ethers and carbamates) would also be suitable. Dipalmityl glycerol was

activated as the acyl carbonyl imidazole (CDI, pyridine) and this activated intermediate was coupled with the module **10** (pyridine, 80°C; 44%). Phosphitylation (CIP(iPr<sub>2</sub>N)OCH<sub>2</sub>CH<sub>2</sub>CN; diisopropylethylamine (DIPEA), CH<sub>2</sub>Cl<sub>2</sub>; 59%) of **16** afforded the phosphoramidite **17** (Scheme 5). Synthesis of a C<sub>18</sub> analog of amidite **17** via a chloroformate intermediate is shown in Scheme 6. The dialkyl glycerol (**18**; DAG) was converted to the corresponding chloroformate **19** upon treatment with excess phosgene in toluene. Conjugation of **19** and amino alcohol **10** was carried out in pyridine to afford adduct **20** in 57% purified yield. Phosphitylation of the secondary hydroxyl of **20** under standard condition afforded phosphoramidite **21** in 95% yield. Coupling of amidite **17** to the 5'-end of a trinucleotide (TTT) on an ABI 394 automated oligonucleotide synthesizer using a slightly modified synthesis cycle with extended coupling times (2x 30 min couplings) for the lipid amidite resulted in 94+% coupling efficiency, as determined by in-line trityl cation analysis.

#### 15 Scheme 5



Scheme 6



5

**Example A- Synthesis of Dipalmitoyl Phosphatidylethanolamine (DPPE)**

**Maleimide Reagent:** A suspension of 200 mg (0.289 mmol) of DPPE and 54 mg (0.347 mmol) of methoxycarbonyl maleimide in 10 mL of THF/ saturated NaHCO<sub>3</sub> solution (1:1)

10 was stirred at ambient temperature. After 12 h, the mixture was treated with 100 mL of EtOAc and the organic phase (which contained a gelatinous suspension of the product) separated from the aqueous phase. The organic phase was concentrated *in vacuo*, coevaporated twice with MeOH, and the resultant white solid triturated three times with EtOAc. This material was used without further characterization or purification in

15 oligonucleotide conjugation experiments (*vide infra*).

**Example B: Synthesis and elaborations of automated Synthesis Module 10.**

**Tetraethylene glycol dimethoxytrityl ether (2):** Tetraethylene glycol (76.4 mL, 0.44 mol) was dissolved in 300 mL of anhydrous pyridine and cooled to 0° C. 4,4'-dimethoxytrityl chloride (15 g, 0.044 mol) was added as a solid with stirring. The reaction flask was covered with a drying tube and the reaction was allowed to warm to ambient temperature overnight. The reaction was concentrated *in vacuo* at low temperature (<30° C). The residue was diluted with 300 mL of ethyl acetate and extracted with 3 x 300 mL

20

of water. The combined aqueous layers were extracted with ethyl acetate, and the combined organic layers were dried over sodium sulfate and concentrated. The crude residue was purified by flash chromatography using 1000 mL of silica gel (wet-packed onto column with hexane containing 5% triethylamine), eluting with 10-20-40-60-80% ethyl acetate in hexane containing 5% triethylamine, and then ethyl acetate containing 5% triethylamine. 19.51 g (89%) of **2** was collected as a gold oil. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 7.47-7.16 (overlapping signals, 9H), 6.79 (d, 4H), 3.72 (s, 6H), 3.66-3.62 (m, 2H), 3.22 (t, J=5.22 Hz, 1H), 2.96 (br t, 1H); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ 158.12, 144.86, 136.04, 129.81, 127.93, 127.49, 126.40, 112.78, 85.67, 72.31, 70.48, 70.44, 70.12, 62.89, 61.39, 54.89; Low resolution MS m/e calculated for C<sub>15</sub>H<sub>25</sub>O<sub>7</sub>S (M-DMT+1<sup>+</sup>): 349.167, found 349.1.

**Tetraethylene glycol dimethoxytrityl ether *p*-toluenesulfonate (**3**):**

Compound **2** ( 5.0 g, 10.06 mmol) was dissolved in 50 mL of anhydrous dichloromethane and cooled to 0° C. Triethylamine (1.82 mL, 13.1 mmol) was added, followed by *p*-toluenesulfonyl chloride (1.92 g, 10.06 mmol) as a solid, with stirring. The reaction was stored in the refrigerator overnight. TLC Analysis indicated the reaction was approximately 80% complete. An additional 0.5 equivalents of triethylamine and 0.5 equivalents of *p*-toluenesulfonyl chloride were added, and the reaction was stirred at room temperature overnight. The reaction was filtered through Celite and concentrated. The residue was purified by flash chromatography using 300 mL of silica gel (wet-packed onto column using 5% triethylamine in hexane) eluting with 25-50-75% ethyl acetate in hexane containing 5% triethylamine, and then ethyl acetate containing 5% triethylamine. 5.7 g (87%) of **3** was collected as an orange oil. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 7.75 (d, 2H), 7.44-7.12 (m, 11H), 6.78 (d, 4H), 4.12-4.09 (m, 2H), 3.73 (s, 6H), 3.66-3.54 (m, 13H), 3.22 (t, J=3.87 Hz, 2H), 2.41 (s, 3H).

**Tetraethylene glycol monotosylate (**2a**):** Tetraethylene glycol (200 mL, 1.15 mol) was dissolved in 500 mL of pyridine and cooled to 0° C and treated with 22.0 g (0.115 mol) of *p*-toluenesulfonyl chloride. When solution was complete, the reaction mixture was stored in the refrigerator overnight, and then concentrated *in vacuo*. The residue was dissolved in 800 mL of EtOAc and extracted with 3 x 600 mL of H<sub>2</sub>O. The H<sub>2</sub>O fractions were back-extracted with EtOAc, and the combined EtOAc fractions were



extracted with saturated aqueous  $\text{Na}_2\text{HPO}_4$ . The organic phase was dried over  $\text{MgSO}_4$  and concentrated to a colorless oil. The oil was purified by flash chromatography using 800 mL of silica gel and eluting with hexane, 25% EtOAc-50% EtOAc in hexane, then EtOAc, then 10% MeOH-20% MeOH in EtOAc to afford 23.7 g (60%) of pure product and 11% of product containing a minor impurity. **2a**:  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  7.77 (d,  $J=8.1$  Hz, 2H), 7.32 (d,  $J=8.1$  Hz, 2H), 4.13 (t,  $J=4.8$  Hz, 2H), 3.68-3.53 (m, 14H), 2.58 (t,  $J=5.6$  Hz, 1H), 2.42 (s, 3H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  168.2, 158.3, 144.8, 135.9, 133.8, 132.0, 129.9, 128.0, 127.7, 126.6, 123.1, 113.0, 85.9, 73.0, 70.6, 70.4, 70.0, 69.7, 67.8, 64.4, 55.1, 37.1; Low resolution MS  $m/e$  calculated for  $\text{C}_{15}\text{H}_{24}\text{O}_8\text{S}$  (M+1): 349.1.

**Tetraethylene glycol monophthalimide (3a)**: To a stirred solution of 31.96 g (0.092 mol) of **2a** in 400 mL of anhydrous DMF was added 14.2 g (1.05 equiv.) of phthalimide and 14.4 mL (1.05 equiv.) of 1,8-diazabicyclo[5.4.0]undec-7-ene. The solution was heated at  $70^\circ\text{C}$  for 18 h then concentrated *in vacuo*. The crude yellow oil was purified by flash chromatography using 1600 mL of silica gel and eluting with 25% EtOAc-50% EtOAc-75% EtOAc in hexane, then EtOAc, then 10% MeOH-20% MeOH in EtOAc to afford 23.8 g (80%) of **3a** as an oil. Upon standing, **3a** became a waxy white solid.  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  7.84-7.78 (m, 2H), 7.70-7.66 (m, 2H), 3.86 (t,  $J=5.6$  Hz, 2H), 3.70 (t,  $J=5.6$  Hz, 2H), 3.64-3.51 (m, 12H), 2.67 (bs, 1H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  168.2, 133.8, 132.0, 123.1, 72.4, 70.5, 70.4, 70.2, 70.0, 67.8, 61.6, 37.2.

**Synthesis of compound 4a**: A solution of 15 g (0.0464 mol) of **3a** in 150 mL of THF and 15 mL of DMF was cooled to  $0^\circ\text{C}$  under Ar. Allyl bromide (6.0 mL, 1.5 equiv.) was added to the solution, followed by addition of 1.76 g (1.5 equiv.) of NaH as a solid. The opaque yellow suspension was stirred at  $0^\circ\text{C}$  for 30 minutes and then at room temperature for 18 hr. MeOH (50-100 mL) was added and concentrated then mixture was concentrated *in vacuo*. The crude material was purified by flash chromatography using 1500 mL of silica gel and eluting with 25% EtOAc-50% EtOAc-75% EtOAc in hexane, then EtOAc, then 10% MeOH in EtOAc to afford 11.05 g (65%) of **4a** as a yellow oil.  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  7.84-7.80 (m, 2H), 7.72-7.67 (m, 2H), 5.94-5.84 (m, 1H), 5.28-5.14 (m, 2H), 3.99 (d,  $J=5.61$  Hz, 2H), 3.88 (t,  $J=5.85$  Hz, 2H), 3.72 (t,  $J=5.76$  Hz,

2H), 3.64-3.54 (m, 13H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ ) d 168.0, 134.6, 133.7, 131.9, 123.0, 116.9, 72.0, 70.4, 69.9, 69.2, 67.7, 37.0.

**(S)-(+)-2,2-Dimethyl-1,3-dioxolanyl-4-ylmethyl (dimethoxytrityl)tetraethylene glycol (5):** Sodium hydride (0.56 g, 23.5 mmol) is weighed into a flame-dried flask, and 70 mL of anhydrous tetrahydrofuran and 15 mL of anhydrous N,N-dimethylformamide were added. The suspension was cooled to 0° C under argon, and (S)-(+)-2,2-dimethyl-1,3-dioxolane-4-methanol (2.7 mL, 21.7 mmol) was added dropwise via syringe. After stirring for 30 min at 0° C, compound **3** (11.77 g, 18.1 mmol) in 15 mL of tetrahydrofuran was added dropwise via addition funnel. The reaction mixture was stirred at ambient temperature overnight, then quenched with 100 mL of saturated aqueous sodium bicarbonate and diluted with 300 mL of diethyl ether. The layers were separated, and the ether layer was extracted 3 times with 300 mL of water. The ether layer was dried over sodium sulfate and concentrated. The residue was purified by flash chromatography using 500 mL of silica gel and eluting first with hexane and then 10-20-30-40-50-75% ethyl acetate in hexane and then with ethyl acetate. 8.93 g (82 %) of **5** was collected as a colorless oil.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ) d 7.46-7.43 (m, 2H), 7.34-7.17 (m, 7H), 6.78 (d, 4H), 4.23 (pentet,  $J=6.1$  Hz, 1H), 4.00 (t, 8.2H), 3.75 (s, 6H), 3.71-3.60 (m, 15H), 3.53 (dd,  $J=10.0, 5.7$  Hz, 1H), 3.52 (10.4,  $J=5.2$  Hz, 1H), 1.39 (s, 3H), 1.33 (s, 3H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ ) d 158.24, 144.97, 136.20, 129.93, 128.07, 127.61, 126.51, 112.89, 109.21, 85.77, 74.57, 72.20, 70.82, 70.59, 70.40, 69.68, 66.67, 63.01, 55.04, 26.67, 25.29; Low resolution MS  $m/e$  calculated for  $\text{C}_{35}\text{H}_{50}\text{O}_9\text{N}$  ( $\text{M}+\text{NH}_4^+$ ): 628.399, found 628.5.

**(S)-(+)-2,2-Dimethyl-1,3-dioxolanyl-4-ylmethyl tetraethylene glycol (6):** 100 mL of 80% acetic acid was cooled to 0° C and then added to compound **5** (6.6 g, 10.8 mmol). The clear orange solution was stirred at 0° C for 1 hr. Methanol (100 mL) was added, and the reaction mixture was concentrated *in vacuo* at low temperature (<30° C). The residue was purified by flash chromatography using 200 mL of silica gel, eluting first with ethyl acetate, and then 5-10-15-20% methanol in ethyl acetate. 2.5 g (74%) of **6** was collected as a colorless oil.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ) d 4.21 (pentet,  $J=6.2$  Hz, 1H), 4.08 (dd,  $J=5.9, 4.8$  Hz, 1H), 3.82-3.35 (m, 19H), 2.93 (br s, 1H), 1.34 (s, 3H), 1.28 (s, 3H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ ) d 109.20, 74.50, 72.42, 72.14, 70.76, 70.39, 70.32, 70.14, 66.63,

61.48, 26.61, 25.23; Low resolution MS m/e calculated for  $C_{35}H_{50}O_9N$  ( $M+NH_4^+$ ): 628.399, found 628.5.

**(S)-(+)-2,2-Dimethyl-1,3-dioxolanyl-4-ylmethyl (phthalimido)tetraethylene glycol (8):** Alcohol 6 (4.06 g, 13.2 mmol) was dissolved in 50 mL of anhydrous

- 5 dichloromethane and cooled to 0° C. Triethylamine (3.7 mL, 26.3 mmol) was added, followed by addition of p-toluenesulfonyl chloride (3.26 g, 17.1 mmol). The reaction flask was covered by a drying tube and allowed to warm to room temperature overnight. The reaction mixture was filtered through Celite, and the filtrate was concentrated *in vacuo*. The crude material was purified by flash chromatography on 400 mL of silica gel, eluting
- 10 first with 10% ethyl acetate in hexane, and then 20-40-60-80-100% ethyl acetate, and then 10% methanol in ethyl acetate. Collected 5.21 g (85%) of the intermediate tosylate as a gold oil. ( $^1H$  NMR (400 MHz,  $CDCl_3$ )  $\delta$  7.79 (d,  $J=8.1$  Hz, 2H, tosyl aromatics), 7.32 (d,  $J=8.1$  Hz, 2H, tosyl aromatics), 4.25 (pentet,  $J=6.0$  Hz, 1H), 4.13 (t, 4.7H), 4.02 (dd,  $J=8.12$ , 6.4 Hz, 1H), 3.71-3.40 (m, 18H), 2.42 (s, 3H), 1.46 (s, 3H), 1.34 (s, 3H);  $^{13}C$
- 15 NMR (75 MHz,  $CDCl_3$ )  $\delta$  144.74, 133.1, 129.76, 127.91, 109.8, 74.63, 72.27, 70.89, 70.68, 70.52, 70.44, 69.19, 68.60, 66.74, 64.1, 26.73, 25.34, 21.59; Low resolution MS m/e calculated for  $C_{21}H_{38}O_9NS$  ( $M+NH_4^+$ ): 480.364, found 480.2.) The tosylate (5.2 g, 11.24 mmol) was dissolved in 60 mL of anhydrous dimethylformamide. 1,8-Diazabicyclo-[5.4.0]undec -7-ene (1.7 mL, 11.24 mmol) was added, followed by
- 20 phthalimide (1.65 g, 11.24 mol). The reaction mixture was heated to 70° C overnight. The reaction was concentrated *in vacuo*, and purified by flash chromatography on 400 mL of silica gel, eluting with 50% ethyl acetate in hexane. Collected 3.96 g (81%) of **8** as a colorless oil.  $^1H$  NMR (300 MHz,  $CDCl_3$ )  $\delta$  7.83-7.79 (m, 2H), 7.72-7.68 (m, 2H), 4.26 (pentet,  $J=6.0$  Hz, 1H), 4.03 (dd,  $J=8.2$ , 6.5 Hz, 1H), 3.88 (t,  $J=5.8$  Hz, 1H), 3.74-3.44
- 25 (m, 18H), 1.39 (s, 3H), 1.33 (s, 3H);  $^{13}C$  NMR (75 MHz,  $CDCl_3$ )  $\delta$  168.21, 133.88, 132.10, 123.19, 109.33, 74.66, 72.30, 70.90, 70.52, 70.04, 67.87, 66.79, 37.19, 26.75, 25.37; MS m/e calculated for  $C_{22}H_{35}O_8N_2$  ( $M+NH_4^+$ ): 455.288, found 455.2.

**1-Dimethoxytrityl-3-(phthalimidotetraethylene glycolyl)-sn-glycerol (9):**

- According to Scheme 2, compound **9** is synthesized as follows: the acetyl **8** (5.16g, 11.8
- 30 mmol) was dissolved in 100 mL of anhydrous methanol, and anhydrous p-toluenesulfonic

acid (100 mg) was added. The reaction flask was covered with a drying tube and the reaction was stirred at ambient temperature for 2.5 h, then neutralized by the addition of 10 mL of anhydrous pyridine, concentrated *in vacuo*, and coevaporated with anhydrous pyridine. The resulting diol was then dissolved in 150 mL of anhydrous pyridine and

5 cooled to 0° C. 4,4'-Dimethoxytrityl chloride (4.39 g, 13 mmol) was added as a solid. The reaction flask was covered with a drying tube, and the reaction was allowed to warm to ambient temperature overnight. Methanol (50 mL) was added, and the reaction was concentrated *in vacuo*. The crude material was purified by flash chromatography on 700 mL of silica gel (wet-packed onto column with 5% triethylamine in hexane), eluting first

10 with 10% ethylacetate in hexane (containing 5% triethylamine) and then 20-40-60-80-100% ethyl acetate (containing 5% triethylamine). Collected 6.98 g (82%) of **9** as a pale yellow oil. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 7.80 (dd, J=5.4, 3.1 Hz, 2H), 7.68 (dd, J=5.4, 3.1 Hz, 2H), 7.42-7.14 (m, 9H, DMT), 6.79 (d, 4H, DMT), 3.95 (br m, 1H), 3.86 (t, J=5.9 Hz, 1H), 3.75 (s, 6H), 3.70 (t, J=5.6 Hz, 1H), 3.63-3.37 (m, 18H), 3.16 (m, 2H),

15 2.84 (br d, 1H); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ 168.15, 158.32, 144.79, 135.95, 133.82, 132.02, 129.95, 128.04, 127.69, 126.64, 123.12, 112.97, 85.89, 72.97, 70.64, 70.43, 69.97, 69.74, 67.80, 64.34, 55.10, 37.14; Low resolution MS m/e calculated for C<sub>40</sub>H<sub>49</sub>O<sub>10</sub>N<sub>2</sub> (M+NH<sub>4</sub><sup>+</sup>): 717.398, found 717.5. According to **Scheme 3**, compound **9** was synthesized as follows: To a stirred solution of **4a** (10.13 g, 0.0279 mol) in 100 mL of

20 acetone and 1 mL of H<sub>2</sub>O was added 3.98 g (1.22 equiv.) of N-methylmorpholine N-oxide. To this suspension was added 1.75 mL (0.005 equiv.) of Osmium tetroxide as a 2.5% solution in iPrOH. After addition of the OsO<sub>4</sub> solution, the reaction mixture became clear yellow. After TLC analysis indicated complete conversion of **4a** (ca 16 h), the reaction mixture was treated with 1.5 g of sodium hydrosulfite and 5.0 g of florisil and stirred 30

25 minutes. The suspension was filtered through florisil, the filtrate was concentrated to an oil. This crude product was combined with another batch prepared in the same manner from 1.0 g of **4a**. Two 100 mL portions of pyridine were co-evaporated from the combined lots and the residue was dissolved in 300 mL pyridine. The solution was cooled to 0° C and 10.89 g (1.05 equiv.) of 4,4'-dimethoxytrityl chloride was added. A drying

30 tube was inserted in the flask and the reaction mixture was stirred at room temperature 16 h. The solution was treated with 20 mL of MeOH and concentrated *in vacuo*, keeping the

temperature of the water bath below 40° C. The crude oil was purified by flash chromatography using 1100 mL of silica gel (wet-packed onto column using 3% triethylamine in hexane) and eluting with 10-100% EtOAc in hexane (all containing 3% triethylamine) to give 21.3 g (89% after two steps) of **9** as a yellow oil. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 7.80-7.77 (m, 2H), 7.66-7.64 (m, 2H), 7.39-7.22 (m, 9H), 7.20-6.76 (m, 4H), 3.97 (bs, 1H), 3.84 (t, J=5.97 Hz, 2H), 3.74 (s, 6H), 3.68 (t, J=5.7 Hz, 2H), 3.60-3.49 (m, 14H), 3.13-2.76 (m, 2H), 2.00 (bs, 1H); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ 168.2, 158.3, 144.8, 135.9, 133.8, 132.0, 129.9, 128.0, 127.7, 126.6, 123.1, 113.0, 85.9, 73.0, 70.6, 70.4, 70.0, 69.7, 67.8, 64.4, 55.1, 37.1; Low resolution MS m/e calculated for C<sub>40</sub>H<sub>45</sub>O<sub>10</sub>N (M+NH<sub>4</sub><sup>+</sup>): 717.5.

**1-Dimethoxytrityl-3-(aminotetraethylene glycolyl)-sn-glycerol (10):**

According to **Scheme 2**, compound **10** was synthesized as follows: Compound **9** (5.2 g, 7.2 mmol) was taken up in 50 mL of 40% methylamine in H<sub>2</sub>O and 10 mL of methanol was added to solublize the starting material. The reaction mixture was heated at 50°C for 5 hr, and then was concentrated *in vacuo* and coevaporated with toluene. The crude material was purified by flash chromatography on 200 mL of silica gel, eluting with 15% methanolic ammonia in dichloromethane. Collected 3.94g (96%) of **10** as a pale yellow oil. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 7.46-7.21 (m, 9H, DMT), 6.81 (d, 4H, DMT), 4.00 (m, 1H), 3.80 (s, 6H), 3.70-3.49 (overlapping m, 18H), 3.20 (dd, J=9.24, 5.49 Hz, 1H), 3.12 (dd, J=9.21, 6.0 Hz, 1H), 2.84-2.80 (m, 3H); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ 158.30, 144.82, 136.01, 129.95, 128.04, 127.66, 126.61, 112.95, 85.85, 73.46, 72.85, 70.55, 70.45, 69.99, 69.51, 64.43, 55.10, 41.40; Low resolution MS m/e calculated for C<sub>32</sub>H<sub>44</sub>O<sub>8</sub>N (M+1<sup>+</sup>): 570.353, found 570.4.

According to **Scheme 3**, compound **10** was synthesized as follows: Compound **9** (5.2 g, 7.2 mmol) was taken up in 50 mL of 40% methylamine in H<sub>2</sub>O and 10 mL of methanol was added to solublize the starting material. The reaction mixture was heated at 50°C for 5 hr, and then was concentrated *in vacuo* and coevaporated with toluene. The crude material was purified by flash chromatography on 200 mL of silica gel, eluting with 15% methanolic ammonia in dichloromethane. Collected 3.94g (96%) of **10** as a pale yellow oil. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 7.46-7.21 (m, 9H, DMT), 6.81 (d, 4H, DMT),

4.00 (m, 1H), 3.80 (s, 6H), 3.70-3.49 (overlapping m, 18H), 3.20 (dd,  $J=9.24, 5.49$  Hz, 1H), 3.12 (dd,  $J=9.21, 6.0$  Hz, 1H), 2.84-2.80 (m, 3H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ ) d 158.30, 144.82, 136.01, 129.95, 128.04, 127.66, 126.61, 112.95, 85.85, 73.46, 72.85, 70.55, 70.45, 69.99, 69.51, 64.43, 55.10, 41.40; Low resolution MS  $m/e$  calculated for  $\text{C}_{32}\text{H}_{44}\text{O}_8\text{N}$  ( $M+1^+$ ): 570.353, found 570.4.

**PEG Reagent 12:** To a stirred solution of 0.24 g (0.41 mmol) of **10** in 10 mL DMF was added 2.08 g (0.4 mmol) of methoxy-PEG<sub>5000</sub>-nitrophenyl carbonate (Shearwater Polymers, Inc.). The mixture was stirred 70 h then concentrated *in vacuo*. The residue was dissolved in EtOAc and the organic phase washed with three 30 mL portions of 10% NaOH solution. Purification by flash chromatography using 100 mL of silica gel (wet packed with dichloromethane containing 5%  $\text{Et}_3\text{N}$ ) eluting with 5-10-15-20% methanolic ammonia in dichloromethane afforded 1.97 g (85%) of **12** as a white solid.  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ ) d 7.42-7.39 (d, 2H, DMT), 7.39-7.18 (m, 7H, DMT), 6.79 (d, 4H, DMT), 5.70 (br m, 1H), 4.21 (m, 1H), 3.97 (m, 1H), 3.88 (t,  $J=4.4$  Hz, 1H), 3.81 (s, 6H, DMT), 3.78-3.50 (br m, ~500, PEG Hs), 3.42-3.31 (overlapping ms), 3.35 (s, PEG Me), 3.16 (m, 2H), 2.84 (br d, 4.2H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ ) d 159.36, 157.19, 146.15, 136.95, 130.83, 128.86, 128.65, 127.61, 113.86, 86.48, 73.58, 72.88, 72.5-70.0 (PEG carbons), 68.31, 65.77, 64.45, 41.36, 30.75 (unassigned impurity).

**PEG Phosphoramidite Reagent 13:** To a stirred solution of 2.22 g (0.4 mmol) of **12** in 60 mL of THF over 3 Å molecular sieves was added 0.24 mL (1.39 mmol) of diisopropylethylamine and 0.1 mL (0.44 mmol) of 2-cyanoethyl N,N-diisopropylchlorophosphoramidite. After 5 h,  $^{31}\text{P}$  NMR indicated formation of desired product, as well as hydrolyzed phosphitylating agent and an additional 0.05 mL (0.22 mmol) of 2-cyanoethyl N,N-diisopropylchlorophosphoramidite was added. After 12 h, 0.07 mL (0.4 mmol) of DIPEA and 0.1 mL of the chlorophosphoramidite were added. The mixture was stirred 2 days, filtered through Celite and concentrated *in vacuo*. The residue was triturated with several portions of ether.  $^{31}\text{P}$  NMR d 156.4, 155.8. Also observed were signals at d 20.6, 19.8 presumed to correspond to hydrolyzed phosphitylation reagent.

**PEG H-Phosphonate Reagent 14:** To a stirred,  $0^\circ\text{C}$  solution of anhydrous imidazole (0.4 g; 5.9 mmol) in 10 mL of MeCN was added 0.17 mL (1.9 mmol) of  $\text{PCl}_3$ ,

followed by 0.86 mL (6.2 mmol) of Et<sub>3</sub>N. To this mixture was added a solution of 3.0 g (0.55 mmol) of **12** in 12 mL of MeCN dropwise. The reaction mixture was allowed to warm to ambient temperature and stirred 16 h, treated with 10 mL of 0.1 M triethylammonium bicarbonate solution, and concentrated *in vacuo*. Triethylamine then  
5 toluene were coevaporated from the crude residue, then the product was dissolved in dichloromethane. The organic phase was washed with 1.0 M triethylammonium bicarbonate (TEAB) solution, dried over sodium sulfate, and concentrated. Purification by flash chromatography on 300 mL of silica gel (wet packed with hexane/ Et<sub>3</sub>N (95:5)) eluting with 2-4-6-8-10-12-15% methanolic ammonia in dichloromethane afforded 1.95 g of  
10 product as a white solid. <sup>31</sup>P NMR (CDCl<sub>3</sub>) δ 10.3, 10.2.

**Synthesis of Lipid phosphoramidite 17:** A solution of 540 mg (1 mmol) of 1,2-di-O-palmityl rac-glycerol in 10 mL of pyridine was treated with 195 mg (1.2 mmol) of carbonyldiimidazole and the resulting mixture stirred at ambient temperature overnight. To this mixture was added 570 mg (1 mmol) of the amino alcohol as a solution in 3 mL of  
15 DMF. The mixture was warmed to 40 °C overnight, after which time <sup>1</sup>H NMR analysis of an aliquot from the reaction indicated very negligible formation of product. The mixture was heated to 80 °C for 6 h (ca 1:1 product to starting material by <sup>1</sup>H NMR), then concentrated in vacuo. The crude residue was applied to a column of 125 mL of SiO<sub>2</sub> gel (Packed in hexanes) and the product eluted with a gradient of 20-50% EtOAc in hexanes  
20 (with 2% TEA) to afford 500 mg (44%) of intermediate **16** as a clear wax. (**16**: <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 7.42 (d, J=7.2 Hz, 2H), 7.30-7.18 (m, 7H), 6.76 (d, J=8.2 Hz, 4H), 5.34 (br t, 1H), 4.20-3.25 (overlapping signals), 3.16 (m, 2H), 1.53 (m), 1.24 (m), 0.86 (t, J=6.5 Hz, 6H). Alcohol **16** (500 mg; 0.44 mmol) was dissolved in 4 mL of CH<sub>2</sub>Cl<sub>2</sub> and 0.15 mL (2 equiv) of DIPEA. To this solution was added 0.15 mL (0.66 mmol) of 2-  
25 cyanoethyl (N,N-diisopropylamino) chlorophosphoramidite. After 3 h, TLC showed conversion to 2 spots and the mixture was diluted with CH<sub>2</sub>Cl<sub>2</sub> and washed with NaHCO<sub>3</sub> solution. The organic phase was dried over Na<sub>2</sub>SO<sub>4</sub> and concentrated. The crude residue was applied to a column of 50 mL of SiO<sub>2</sub> gel (packed in hexanes) and the product eluted with 20 % EtOAc in hexanes (containing 2% TEA) to afford 350 mg (59%)  
30 of phosphoramidite **17** as a colorless wax. <sup>31</sup>P NMR (CDCl<sub>3</sub>) δ 151.55, 151.08.

**Automated synthesis of lipid-oligo:** Phosphoramidite **17** was coupled to the 5'-end of a T-3mer (prepared by standard automated DNA synthesis on an ABI 394 instrument) using a modified coupling cycle consisting of two 30 minute exposures of an 0.1 M solution of **17** in 40% THF in MeCN to the column. In-line trityl analysis indicated a coupling efficiency of 94% for amidite **17**.

**Chloroformate 19:** To a stirred solution of 3 g (5.03 mmol) of 1,2-di-*O*-octadecyl-*sn*-glycerol **21** in 60 mL of toluene was added 20 mL of a 1.93 M solution of phosgene. Additional phosgene solution (2 X 10 mL; 15.4 equiv phosgene total) was added until no further alcohol starting material remained (by <sup>1</sup>H NMR analysis of concentrated aliquots). The excess phosgene and HCl was removed by aspirator and the reaction mixture was concentrated *in vacuo* to afford 3.3 g (98%) of the desired chloroformate **19** as a white powder. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 4.45 (dd, J=11.22, 3.69 Hz, 1H), 4.34 (dd, J=11.22, 6.15 Hz, 1H), 3.65 (m, 1H), 3.56-3.40 (m, 6H), 1.53 (m, 4H), 1.24 (m, 62H), 0.87 (t, J=6.36 Hz, 6H); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ 75.90, 71.91, 71.35, 70.93, 69.36, 31.99, 29.96-29.44 (overlapping signals from hydrocarbon chains), 26.13, 26.04, 22.76, 14.18.

**Conjugate 20:** To a stirred solution of 2.25 g (3.95 mmol) of **10** in 60 mL of pyridine was added 2.6 g of the distearyl glycerol chloroformate **18**. <sup>1</sup>H NMR analysis of a concentrated aliquot after 2 h revealed no remaining chloroformate and the mixture was concentrated *in vacuo*. The crude residue was combined with material similarly prepared from 0.5 g (0.88 mmol) of **10** and 0.58 g of the chloroformate and the combined lots purified by flash silica gel chromatography on a column of 100 mL of silica gel (packed in hexanes containing 2% triethylamine) eluting with 200 mL hexanes, then 250 mL each of 10-20 and 30% EtOAc in hexanes, 500 mL 40% EtOAc in hexanes, then 250 mL each of 50-60-70 and 80% EtOAc in hexanes, and finally with 250 mL of EtOAc. The product containing fractions were concentrated to afford 3.3 g (57%) of the conjugate **20**.

**Phosphoramidite 21:** To a stirred solution of 3.8 g (3.26 mmol) of the conjugate in 25 mL of CH<sub>2</sub>Cl<sub>2</sub> was added 1.14 mL (6.52 mmol) of diisopropylethylamine then 1.09 mL (4.88 mmol) of 2-cyanoethyl N,N-diisopropylchloro-phosphoramidite. After 2 hours, the mixture was diluted with CH<sub>2</sub>Cl<sub>2</sub> and washed with saturated NaHCO<sub>3</sub> solution, dried over Na<sub>2</sub>SO<sub>4</sub>, and concentrated. The crude residue was purified by flash silica gel chromatography on a column of 125 mL of silica gel (packed in hexanes containing 2%



triethylamine) eluting with 100 mL hexanes, then 250 mL each of 10 and 20% EtOAc in hexanes, 500 mL 30% EtOAc in hexanes, then 250 mL of 50% EtOAc in hexanes. The product containing fractions were concentrated to afford 4.2 g (95%) of the phosphoramidite 21.  $^{31}\text{P}$  NMR ( $\text{CDCl}_3$ )  $\delta$  151.52, 151.08.

5

**EXAMPLE 2. PREPARATION AND FUNCTIONAL PROPERTIES OF PEG CONJUGATED AND CHOLESTEROL-DERIVATIZED NUCLEIC ACID LIGANDS.**

A PEG 3400 conjugate of a Nucleic Acid Ligand retains the binding affinity of the non-conjugated molecule

10       The ability of a bFGF ligand/PEG-3400 conjugate to bind bFGF was tested. Molecule 225t3 (SEQ ID NO:10), a high affinity DNA ligand to bFGF, was chosen for conjugation with PEG via a primary amine-NHS coupling reaction. Ligand 225t3 has a binding affinity of 1 nM and folds into a blunt ended hairpin with a  $T_m$  of 68 °C. Ligand 225t3 was modified with a 3'-amino-modifier C7 CPG (Glen Research, Sterling, VA) using  
15       standard DNA synthesis methods and will be referred to as 225t3N (SEQ ID NO:11). 225t3N was reacted with the N-hydroxysuccinimide (NHS) active ester of PEG (avg. MW 3400) in 20% (v/v) dimethoxy formamide 80% (v/v) 0.5 M sodium bicarbonate buffered at pH 8.5. The resulting conjugate, 225t3N-PEG-3400 (SEQ ID NO:14), was purified from the free DNA on a 12% polyacrylimide/7 M urea gel. The conjugate was 5'  
20       end-labeled with  $^{32}\text{P}$  and a binding assay was performed. 225t3N-PEG-3400 (SEQ ID NO:14) binds to bFGF with the same affinity ( $K_d=1$  nM) as 225t3.

Conjugation of PEG-20.000 to a thrombin DNA ligand.

25       Thrombin DNA ligand NX256 (SEQ ID NO:9) (Figure 1D) containing an amino and a disulfide functionality was prepared using standard DNA synthesis methods and procedures on a Biosearch 8909 DNA/RNA synthesizer with dT-5'-LCAA-500 Å controlled-pore glass solid support and commercially available phosphoramidite reagents. Deprotection was followed by ion exchange HPLC purification. The 5' terminal disulfide bond was reduced by incubating the DNA in a solution 50 mM dithiothreitol (DTT) at 37°C for 30 min.. The reduced DNA (containing a 5' terminal thiol) was run through a  
30       Nap-5 size exclusion column and the void volume, containing the DNA but not the DTT, was collected into a reaction vessel containing the maleimide derivatized PEG under an argon blanket. All solutions were purged with argon to remove oxygen. The reaction was kept at 40°C for 1 h. The progress of the reaction was monitored by removing small

aliquots and analyzing them by electrophoresis on 8%/7 M urea polyacrylamide gels. At the end of the 1 hour incubation period, the reaction was essentially complete, and at this time an equal volume of methylene chloride was added to the reaction mix and the vessel shaken until a milky white suspension formed. The mix was spun at 14,000 rpm in an eppendorf centrifuge until the layers separated. The aqueous layer contained the free Nucleic Acid Ligand and was discarded. The product (PEG-20,000 modified DNA ligand NX256, referred to as NX256-PEG-20,000; SEQ ID NO:13) (Figure 1G) was further purified using ion exchange chromatography followed by reverse phase desalting and lyophilization to a white powder. This material was used to determine the effect of PEG modification on the pharmacokinetic behavior of the DNA Nucleic Acid Ligand (*vide infra*). PEG-10,000 modified ligand NX256, referred to as NX256-PEG-10,000, was prepared in an analogous manner.

#### T<sub>m</sub> Values for PEG conjugates

PEG functionality can also be introduced via the thiophosphate-maleimide reaction. We introduced the thiophosphate group into the thrombin DNA ligand at the 5'-end by the standard phosphoramidite method using commercially available reagents (this ligand is referred to as T-P4) (Figure 1F, SEQ ID NO:12). The conjugation to the maleimide-containing PEG is analogous to the sulfhydryl-maleimide reaction described above. The PEG-conjugated ligands are referred to as T-P4-PEG-10,000 and T-P4-PEG-20,000 (SEQ ID NO:15). Melting temperatures (T<sub>m</sub>) for T-P4-PEG-10,000, T-P4-PEG-20,000 and T-P4-DNA ligands were determined from the first derivative of the optical absorbance at 260 nm vs. temperature plots. The T<sub>m</sub> values for T-P6-PEG-10,000, T-P6-PEG-20,000 and T-P6 were 40 °C for all three ligands. These data and the bFGF-PEG-3400 Nucleic Acid Ligand binding data reported above, suggest that conjugation to PEG does not affect Nucleic Acid Ligand structure.

#### A Cholesterylated bFGF ligand retains the binding affinity of the non-cholesterylated molecule

Cholesterol can be introduced into a Nucleic Acid Ligand, at any position in the sequence, by the standard solid phase phosphoramidite method. For example, we incorporated a tetraethyleneglycol cholesterol phosphoramidite (Glen Research, Sterling, VA) at the 3' end of ligand 225t3 (Figure 1E; SEQ ID NO:10) to produce ligand 225t3-Cholesterol. Following purification on a 12% polyacrylimide/7 M urea gel, 225t3-Chol

was 5' end-labeled with  $^{32}\text{P}$  and a binding assay performed. The binding affinity of 225t3-Chol was identical ( $K_d=1$  nM) to that of 225t3.

### **EXAMPLE 3 NUCLEIC ACID LIGAND-LIPOSOME FORMULATION AND ANTICOAGULATION ACTIVITY.**

#### **A. Preparation of NX232 Liposomes**

Fluorescein-labeled, cholesterol-derivatized NX232 (Figure 1B; SEQ ID NO:7) was incorporated into Liposomes composed primarily of distearoylphosphatidylcholine (DSPC) and cholesterol (Chol) in a 2:1 molar ratio.

Eight formulations of NX232 Liposomes containing DSPC:Chol (2:1 mole ratio) were prepared. The mole percentage of NX232 was varied from 0.01 through 0.1 mole %, based upon total lipids present. The compositions (A-H) are reported in Table 1. Increasing fractions of the cationic lipid 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) were included in formulations D-H to evaluate the effect of positive charges on the strength of the association between NX232 and the Liposome surface.

**TABLE 1. Summary of Liposome NX232 Preparations - Lipid compositions and mole percentage of NX232**

Compound <sup>a</sup> M.W.	A	B	C	D	E	F	G	H
mole fractions:								
DSPC 790.15	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Chol 386.7	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
DOTAP 698.55	0.0	0.0	0.0	0.0	0.0012	0.003	0.006	0.012
NX232 12,424.1	0.0003	0.0008	0.0015	0.003	0.0003	0.0008	0.0015	0.003
(NX232 mole-%)	0.01	0.025	0.05	0.1	0.01	0.025	0.05	0.1
Weight Ratios:								
DSPC	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Chol	0.2447	0.2447	0.2447	0.2447	0.2447	0.2447	0.2447	0.2447
DOTAP	0.0	0.0	0.0	0.0	0.0005	0.0013	0.0027	0.0053
NX232	0.0024	0.0059	0.0118	0.0236	0.0024	0.0059	0.0118	0.0236
Amounts per ml <sup>b</sup> :								
DSPC	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Chol	4.894	4.894	4.894	4.894	4.894	4.894	4.894	4.894
DOTAP	0.0	0.0	0.0	0.0	0.011	0.027	0.053	0.106
NX232	0.047	0.118	0.236	0.472	0.047	0.118	0.236	0.472

<sup>a</sup> DSPC = distearoylphosphatidylcholine; Chol = cholesterol; DOTAP = 1,2-dioleoyl-3-trimethylammonium-propoane.

<sup>b</sup> final total concentration for all components = 25 mg/ml.

Lipids and NX232 were co-solublized in a mixture of chloroform, methanol and water ( $\text{CHCl}_3:\text{MeOH}:\text{H}_2\text{O}$ , 1:5:1, v:v:v) and transferred to test tubes. Lipid films were formed by evaporating the solvent under nitrogen flow. The dried lipid films were stored  
5 under vacuum until hydration. The films were hydrated at  $65^\circ\text{C}$  with an aqueous 9% sucrose solution ( $\sim 250\text{ mM}$ ) containing  $10\text{ mM}$  tris(hydroxymethyl)aminomethane (TRIS) and  $1\text{ mM}$  ethylenediaminetetraacetic acid (EDTA) at pH 7.4. Following hydration, the lipids were sonicated using a probe-type sonicator for approximately 6-9 minutes and then cooled to room temperature.

10 Unincorporated NX232 (Figure 1B; SEQ ID NO:7) was removed by FPLC gel permeation chromatography using Sephacryl S-300, eluting with the sucrose hydrating solution described above. The gel permeation chromatograms for empty Liposomes (Empty), Liposomes with  $4.7\text{ }\mu\text{g}$  NX232 (L-NeX2a), Liposomes with  $11.8\text{ }\mu\text{g}$  NX232 (L-NeX2b), free NX232 at  $72\text{ }\mu\text{g}$  (Free  $72\text{ }\mu\text{g}$ ), free NX232 at  $7.2\text{ }\mu\text{g}$  (Free  $7.2\text{ }\mu\text{g}$ ), and free  
15 NX232 at  $10\text{ }\mu\text{g}$  which had been sonicated (Free/soni  $10\text{ }\mu\text{g}$ ) are shown in Figure 2. The chromatograms indicate that good separation of free NX232 and Liposomes is possible. The free NX232 chromatogram at  $72\text{ }\mu\text{g}$  shows a distinct peak following the Liposomes (elution peak at  $\sim 53$  minutes); however,  $72\text{ }\mu\text{g}$  of the free Nucleic Acid Ligand had to be added in order to be visualized by UV absorbance at  $254\text{ nm}$ . No similar peak can be  
20 discerned for free NX232 at  $7.2\text{ }\mu\text{g}$ . Therefore, with the liposomal preparations, the NX232 amounts ( $4.7\text{ }\mu\text{g}$  for "a" and  $11.8\text{ }\mu\text{g}$  for "b") may have been insufficient to show up as a distinct peak on the chromatogram.

Based on the absorbance measurements at  $254\text{ nm}$ , NX232 is incorporated nearly quantitatively into these Liposomes. Since only approximately 50% of the NX232  
25 molecules were fluorescein labeled, quantitative incorporation suggests that the presence of the fluorescein label does not significantly affect association of NX232 with Liposomes.

#### **B. *In Vitro* Assay of Clotting Inhibition**

NX232 (Figure 1B; SEQ ID NO:7) contains the DNA sequence of a high affinity Nucleic Acid Ligand to thrombin. Thrombin is a critical component of the blood clotting  
30 cascade. The inhibition of the proteolytic activity of thrombin is known to decrease the ability of blood to clot. The activity of the various NX232 formulations were evaluated

using a fibrin/thrombin clotting assay to measure anticoagulation activity. A buffer solution of 50 mM TRIS, 100 mM NaCl, 1 mM MgCl<sub>2</sub>, and 0.1% polyethylene glycol (PEG<sub>8000</sub>) (MW 8,000) at pH 7.4 was used for the assay. In the final 300 µl assay mixture, fibrinogen at a concentration of 2.5 mg/ml and thrombin at 1 National Institutes of Health (NIH) unit were added to glass test tubes. All solutions and containers were warmed to and maintained at 37°C for coagulation measurements. Coagulation times are reported in Table 2.

**Table 2. Effect of Processing on NX232 Anticoagulation Activity**

Preparation	NX232 (µg/300µl)	Clot Time (sec.)
Control (no additions)	---	18-20
NX232, unsonicated	7.08	49-51
	3.54	19-21
NX232, sonicated	7.08	45-50
	3.54	20-25
Liposomes: Preparation A	7.08	57-59
	3.54	26-28
Preparation B	7.08	56-59
	3.54	25-27

These results indicate that typical Liposome processing conditions, including sonication, solubilization, heating and drying, do not affect the anticoagulation activity of NX232. In addition, liposomal association does not affect the ability of NX232 to bind and inhibit its target.

**EXAMPLE 4. PHARMACOKINETIC PROPERTIES OF CHOLESTEROL, DIACYL GLYCEROL, DIALKYL GLYCEROL, AND PEG MODIFIED DNAs.**

The pharmacokinetic properties of thrombin DNA ligands NX229, NX232, NX253, NX253 + Liposome, and NX256-PEG20K were determined (see Figures 1A-1C, 1G for molecular descriptions) (SEQ ID NOS:6-8, 13). Each oligonucleotide was diluted in PBS to a solution concentration of 0.5-1.0 mg/ml based on UV absorption at 260 nm and an extinction coefficient of 0.033 µg oligo/ml. In all but one study, 6 rats received 0.5-1.0 mg oligonucleotide/kg animal weight and plasma samples were taken at various times from

1 minute to 4 hours. One rat was used in the study in which NX253 was tested. The plasma samples and quality control samples were analyzed using a hybridization assay. The hybridization assay utilized a capture oligonucleotide that contains a complementary sequence to the 5'-end of the DNA ligands conjugated to an iron oxide (FeO) bead (FeO-spacer-5'-d (GTC AGG CAC CAT CCC-3') (SEQ ID NO:1) where spacer = (dT)<sub>8</sub>), and a  
5 detection oligonucleotide containing a biotin group at the 3'-end (5'-d-CCC CAC TGA AGC ACC-spacer-3'-biotin-biotin, where spacer = (dT)<sub>10</sub>) (SEQ ID NO:2). The amount of the biotin oligonucleotide attached to the bead was quantitated with the streptavidin-linked alkaline phosphatase, using CSPD-Sapphire as the luminescent substrate.

10 Data for the plasma concentration of NX229, NX232, NX253, NX253 + Liposome, and NX256-PEG20K (SEQ ID NOS:6-8, 13) as a function of time following the bolus injection are summarized in Figure 3. The plasma concentrations of NX232, NX253, NX253 + Liposome, and NX256-PEG20K as a function of time are considerably greater compared to that of NX229 (SEQ ID NO:6). All of these oligonucleotides share  
15 the same thrombin binding module (d(CAG TCC GTG GTA GGG CAG GTT GGG GTG ACT TCG TGG)) (SEQ ID NO:3). The plasma concentration of an oligonucleotide as a function of time can be significantly increased by introducing appropriate functional groups into the oligonucleotide. Prolonged plasma half-life of a cholesterol-containing oligonucleotide compared to the control (non-cholesterol-containing) oligonucleotide has  
20 been observed previously (de Smidt *et al.*, Nucl. Acids Res., 19: 4695 (1991))

The plasma pharmacokinetic properties of a wide number of Nucleic Acid Ligands that have various functional groups attached to the base sequence of NX213 (see Figure 1 for molecular description), as well as some liposomal formulations of these oligonucleotides have been assessed. These data are summarized in Figure 4 (SEQ ID  
25 NOS:16, 19, 21, 22 and 29). The formulation with the slowest clearance rate was where NX213 (Figure 1P; SEQ ID NO:21) was encapsulated within liposomes.

To determine the role of dialkyl glycerol DNA conjugates plus and minus liposomes PK studies 77 and 73 were carried out with the Thrombin DNA ligand dialkyl glycerol conjugate (See Figure 1 for molecular description). These data are summarized in Figure 5  
30 (SEQ ID NO:18).

Cholesterylated VEGF oligonucleotides NX213 (NX268) (See Figure 1K for molecular description; SEQ ID NO:16) were formulated with either PEG-liposomes or

standard liposomes and the pharmacokinetics were evaluated (PK 85-86). These formulations contain oligonucleotides both on the inside and outside of the liposome. Figure 6 (SEQ ID NO:16) shows the rat plasma levels of full length oligonucleotides as a function of time after injection. Both liposome formulations show similar oligonucleotide pharmacokinetics.

To evaluate size dependence on clearance, 2' O-methyl VEGF oligonucleotides with various PEG conjugates (See Figure 1 for molecular description) (PK 50,80,87, &88) have been studied. Figure 7 shows the comparison of the all 2' O-methyl oligonucleotides plus PEG 40K, 20K, 10K, as well as in the absence PEG (SEQ ID NOS:17 and 29). These data demonstrate significantly slower plasma clearance with increasing size of the PEG conjugate.

PK 96 was carried out to evaluate the pharmacokinetics of 2' F pyrimidines in conjunction with 2' O-methyl purines and PEG20K (JW1130) (See Figure 1R for molecular description; SEQ ID NO:23). Figure 8 shows the plasma levels of this oligonucleotide in comparison with the all 2' O-methyl version (PK 80, JW986) (See Figure 1X for molecular description; SEQ ID NO:29). These data show fairly similar clearance properties for both oligonucleotides.

The observation that JW1130 (SEQ ID NO:23), containing 2' F pyrimidines, shows similar clearance to JW986 (2' O-methyl pyrimidines) suggests that oligonucleotides with 2'F pyrimidines are resistant to nuclease digestion.

PK 97 was carried out to determine the clearance properties of a L-Selectin DNA ligand (NX287) (Figure 1S; SEQ ID NO:24) conjugated with 40K PEG. Figure 9 shows the plasma levels of this oligonucleotide as a function of time after bolus injection (dose 1 mg/kg). For comparison, Thrombin DNA ligand (NX256) (Figure 1H; SEQ ID NO:13) conjugated with 20K PEG was included. As shown in Figure 9 these two oligonucleotides show similar clearance rates presumably due to metabolism.

PK studies 99, 100 and 102 have been carried out as part of a larger study to assess the stability of oligonucleotides *in vivo*. These studies are shown in Figure 10. For comparison, PK 96 (JW1130 VEGF) 2'F Py 2'O-Met (14 Pu) + PEG 20K ) (Figure 1R; SEQ ID NO:23) is also included. The oligonucleotides used in PK studies 96, 100, and 102 differ only in the number of purine positions that contain 2' deoxy nucleotides, where PK 96 contains no 2' deoxy purines, PK 100 four 2' deoxy purines, and in PK 102 all 14 purines are 2' deoxy. Figure 10 (SEQ ID NOS:23, 25, 26, 27 and 30) demonstrates a clear

relationship between increasing clearance rate and the number of deoxy nucleotides present in the oligonucleotide. This observed increase in clearance rate with increasing number of deoxy nucleotides is assumed to be due to increased metabolism of these oligonucleotides. An encouraging observation is the high level of stability shown for PK 100 containing four 2' deoxy purines, and suggests that post-SELEX modification may be appropriate if a large number of purines can be modified. Also shown in Figure 10 is PK 99 vs. PK 100, that differ in PEG20K conjugation. As previously observed with other oligonucleotides, conjugation to PEG molecules of significant molecular weight dramatically reduces the observed clearance rate from plasma.

**EXAMPLE 5. CATIONIC LIPOSOME-NUCLEIC ACID LIGAND COMPLEXES: TOXICITY AND THEIR INTRACELLULAR UPTAKE BY HUMAN LYMPHOCYTES.**

**Toxicity.** To determine toxic effects of Liposome-Nucleic Acid Ligands on cells, the human primary peripheral blood lymphocytes (PBLs) were treated with Nucleic Acid Ligand alone, the two types of Liposomes alone, and the two Liposome-Nucleic Acid Ligand combinations (*vide infra*). Two different types of Liposomes, type 1 (=dioleoylphosphatidylethanolamine (DOPE):aminomannose cholesterol at 1:1 weight ratio) and type 2 (=DOPE: aminomannose cholesterol:DOTAP at a 1:1.5:1 weight ratio) were used for this study. The Liposomes were mixed at a ratio of 5:1 (Liposome:Nucleic Acid Ligand, by weight) with single stranded DNA SELEX ligand RT1t49-PS (5'-d(ATC CGC CTG ATT AGC GAT ACT CAG AAG GAT AAA CTG TCC AGA ACT TGG AsTsTsTsT)-3' (SEQ ID NO:4), where lowercase s indicates a phosphorothioate linkage) that binds to HIV-1 reverse transcriptase with a  $K_d$  of approximately 1-5 nM. The Cationic Liposome-Nucleic Acid Ligand Complex was formed by incubating the Liposome and the type 1 or type 2 Liposome with RT1t49-PS at 65°C for 10 minutes.

PBLs (phytohemagglutinin and natural IL-2 stimulated) were plated at a density of  $2 \times 10^5$  cells per well in 96-well plates. Cells are treated at day 0, split and retreated at day 4 with Nucleic Acid Ligand alone, or the two types of Liposomes alone, or the two Liposome-Nucleic Acid Ligand Complexes. The viable cells were counted at day 7. The percent of viable cells for each of the treatment groups is summarized below:



<u>Treatment</u>	<u>% Viability</u>
cells alone	85
30 µg/ml RT1t49PS	82
Liposome type 1 (150 µg/ml)	85
5 Liposome type 2 (150 µg/ml)	63
Liposome type 1 (150 µg/ml) + RT1t49PS (30 µg/ml)	88
Liposome type 2 (150 µg/ml) + RT1t49PS (30 µg/ml)	77

These results suggest the following. Ligand RT1t49PS was not toxic at concentrations up to 30 µg/ml. Liposome type 1 not toxic at concentrations up to 150 µg/ml while Liposome type 2 is moderately toxic (about 25% reduced viability) at this concentration. The toxicity of Liposome type 2 is expected because DOTAP is known to be toxic. Ligand RT1t49PS apparently reduces the toxicity of Liposome type 2 by about ~50%.

**15            Cellular uptake.** Intracellular delivery of fluoresceinated RT1t49PS ligand was examined with CEMss cells (human T cell line) using fluorescence activated cell sorting (FACS) analysis and confocal microscopy. For this study DOPE:aminomannose (1:1 mole ratio) Liposomes were used. Lipid films were prepared from 2.33 mg DOPE and 2.33 mg aminomannose, dissolved in chloroform, and kept under vacuum in a desiccator

20 overnight. 1 ml of 9% sucrose was added to the film and the tube was heated at 65°C for 0.5 minutes and vortexed. The lipid mixture was then sonicated at a power setting of 7 (microtip) for 2 minutes in a beaker containing water heated to 50°C. An additional 0.5 ml of 9% sucrose was added to the Liposomes and the Liposomes were sized using a

25 MicroTrac particle sizer (average size 45 nm) and sterile filtered using a 0.45 µm cellulose acetate filter. Liposome-Nucleic Acid Ligand Complexes were prepared by incubating the Liposome with the Nucleic Acid Ligand at a 5:1 lipid:oligonucleotide w/w ratio for 10 minutes at 65°C.

For the cellular uptake experiment, 10<sup>6</sup> CEM cells were diluted in 10 ml of 1640 RPMI/10% fetal calf serum in T25 flasks. The Nucleic Acid Ligand at 0.6 µM, as free

30 drug or in a Liposome Complex, was added to each flask and incubated at 37°C in an atmosphere containing 5% CO<sub>2</sub>. Prior to observation, cells were centrifuged and washed twice to remove excess drug.

Confocal Microscopy was performed with an air-cooled argon laser (excitation 488nm). Confocal images were taken at 1  $\mu$ m slices (approximately 20 slices per series). Through 24 hours, no significant fluorescence (above background) was detectable in CEM cells incubated with Nucleic Acid Ligand alone. Significant fluorescence was detected in CEM cells incubated with Liposome-associated Nucleic Acid Ligands by 5 hours and increased through 24 hours. Fluorescence appeared to be localized in small vacuoles and not in the nucleus. In polarized cells (example 7 hr incubation with Liposome-associated Nucleic Acid Ligand), the fluorescence is localized in the rear of the cell away from the leading/advancing edge.

FACS analyses were performed with a Coulter Epics Elite equipped with an air-cooled argon laser (excitation 488 nm). CEM cells were gated for forward and side scatter and examined for green fluorescence. Dead cells and aggregates were excluded from the gate. As suggested by confocal microscopy, the fluorescence of cells incubated with Liposome-associated Nucleic Acid Ligand is about an order of magnitude greater than that of cells incubated with free Nucleic Acid Ligand. Uptake of Liposome-associated Nucleic Acid Ligand is not entirely homogeneous. Some cells are significantly more fluorescent than others. For a 5:1 w/w lipid:Nucleic Acid Ligand ratio ( $M_r$  of Nucleic Acid Ligand $\approx$ 14,000;  $M_r$  of lipid $\approx$ 700) and assuming 40,000 lipids per Liposomes, there are approximately 400 Nucleic Acid Ligands per Liposome. The lower detection limit of the FACS is approximately 500 fluorophores per cell or slightly greater than 1 Liposome per cell.

In conclusion, free 5'Fl-RT1t49PS Nucleic Acid Ligands do not significantly localize within CEM cells within 24 hours. Nucleic Acid Ligands associated with DOPE:aminomannose Liposomes localize within CEM cells by 5 hours and continue to localize in the cells through 24 hours. Liposome-associated Nucleic Acid Ligands appear to accumulate in vacuoles and not in the nucleus. The amount of Liposome-associated Nucleic Acid Ligand uptake is at least ten times greater than for free Nucleic Acid Ligand, as judged by FACS analysis.

**EXAMPLE 6. INCORPORATION OF NUCLEIC ACID LIGANDS INTO  
PREFORMED LIPOSOMES: EFFECT OF VARYING THE NEGATIVE  
CHARGE OF THE LIPIDS.**

Small unilamellar vesicles (SUV) composed of distearoylphosphatidylcholine (DSPC), cholesterol (Chol), and distearoylphosphatidylglycerol (DSPG) were prepared using formulations with the molar ratios shown in Table 3. Four compositions containing varying molar percentages of DSPG, a negatively-charged lipid, were prepared to evaluate the effect of negative Liposome charge on the incorporation of a polyanionic Nucleic Acid Ligand. The lipids were dissolved in  $\text{CHCl}_3$ , mixed and dried under a steady stream of nitrogen. The dried lipid film was further dried and stored under vacuum overnight prior to hydration. The lipid film was hydrated with a pH 7.4 phosphate buffer solution (PBS), containing  $\text{Na}_2\text{HPO}_4$  (1.15 g/L),  $\text{NaH}_2\text{PO}_4$  (0.228 g/L), and sucrose (90 g/L), at 65 °C to yield a 50 mg/mL lipid suspension. The hydrated lipid suspension was then sonicated for 15 - 30 min using a probe-type sonicator until an opalescent solution was obtained.

These preformed SUV were added to an equal volume of Nucleic Acid Ligand 232 (NX232) (SEQ ID NO:7), 1.0 mg/mL in PBS (final concentrations: 0.5 mg/mL NX232, 25 mg/mL lipid). The mixture was incubated at 65°C for 15 min or kept at room temperature before being chromatographed on a Sephacryl HR S300 size-exclusion column (0.5 x 20 cm) to separate free from SUV-bound NX232. Chromatography conditions were as follows: eluent, PBS described above; flow rate, 0.1 mL/min; sample injected, 25  $\mu\text{L}$ ; detector, UV absorbance at 254 nm; fraction, 0.2 mL/fraction. The collected fractions were also monitored by fluorescence intensity (excited at 494 nm and emitted at 516 nm).

SUV-associated NX232 eluted with the SUV peak (excluded volume) and free-NX232 eluted in the included volume. The chromatogram (Figure 4) clearly demonstrates that the degree of NX232 association with SUV was dependent upon the DSPG content in the SUV. As the percentage of negatively charged DSPG contained in the SUV was increased between samples A-D, NX232 association with SUV decreased.

**Table 3: Composition of Liposomes with Various Negative Charges**

Lipid	Molar Percentage			
	(A)	(B)	(C)	(D)
DSPC	85	87.5	89	90
Cholesterol	10	10	10	10
DSPG	5	2.5	1	0

**EXAMPLE 7. INCORPORATION OF NUCLEIC ACID LIGANDS INTO**  
**5 PREFORMED LIPOSOMES: EFFECT OF VARYING THE CHOLESTEROL**  
**CONTENT**

SUV composed of DSPC and Chol were prepared and NX232 incorporation  
 assayed as in Example 6. The Liposomes contained different molar ratios of DSPC and  
 cholesterol as indicated in Table 4. NX232 associated with the Liposomes and eluted  
 10 with the SUV when prepared at room temperature.

**Table 4: Composition of Liposomes with Various Cholesterol Contents**

Formulation	Mole % DSPC	Mole % Cholesterol
(E)	95	5
(F)	90	10
(G)	85	15
(H)	80	20
(I)	75	25
(J)	70	30
(K)	65	35

15 Liposome formulations J and K (approximately 2:1 mole ratio of  
 DSPC:cholesterol) allow for the most efficient incorporation of the Nucleic Acid  
 Ligand NX232.

**EXAMPLE 8. INCORPORATION OF NUCLEIC ACID LIGANDS INTO  
PREFORMED LIPOSOMES: EFFECT OF VARYING LIPID/NUCLEIC ACID  
LIGAND RATIO WITH A FIXED AMOUNT OF NX232**

DSPC:Chol (2:1 molar ratio) SUV were prepared and assayed as in Example 6, but with varying lipid/NX232 ratios. A fixed amount of NX232 (SEQ ID NO:7), 1.0 mg/mL, was mixed at room temperature with an equal volume of SUV, containing lipid concentrations from 2.5 to 50 mg/mL (Table 5). The results suggest that the maximal association of NX232 with SUV was achieved at lipid/NX ratios (w/w) of 25/1. The highest lipid/NX ratio, 50/1, did not increase the amount of NX232 bound to SUV.

**Table 5: Lipid/NX Ratios (w/w) Tested with A Fixed Amount of NX232**

<b>Lipid Conc. (mg/mL)</b>	<b>NX Conc. (mg/mL)</b>	<b>Lipid/NX Ratio (w/w)</b>
2.5	1.0	2.5/1
5.0	1.0	5/1
10.0	1.0	10/1
25.0	1.0	25/1
50.0	1.0	50/1

**EXAMPLE 9. INCORPORATION OF NUCLEIC ACID LIGANDS INTO  
PREFORMED LIPOSOMES: EFFECT OF VARYING LIPID/NUCLEIC ACID  
LIGAND RATIO WITH A FIXED AMOUNT OF SUV**

Preformed SUV (DSPC/CH: 2/1), 50 mg/mL, prepared as in Example 6, were mixed with an equal volume of NX232 at various concentrations from 0.5 to 5.0 mg/mL at room temperature (Table 6). The results indicate that maximal association of NX232 with SUV was obtained at a lipid/NX232 ratio (w/w) of 25/1. The fraction of NX232 associated with SUV decreased with lower lipid/NX232 ratio.

**Table 6: Lipid/NX Ratios (w/w) Tested with A Fixed Amount of SUV**

<b>Lipid Conc. (mg/mL)</b>	<b>NX Conc. (mg/mL)</b>	<b>Lipid/NX Ratio (w/w)</b>
50	5.0	10/1
50	4.0	12.5/1
50	3.0	16.7/1
50	2.5	20/1
50	2.0	25/1
50	1.0	50/1
50	0.5	100/1

**EXAMPLE 10. INCORPORATION OF NUCLEIC ACID LIGANDS INTO  
PREFORMED LIPOSOMES: EFFECT OF VARYING THE PHOSPHOLIPID  
CHAIN LENGTH**

SUV were prepared as in Example 6 from the phospholipids indicated in Table 7 to study NX232 association with SUV made of phospholipids with different chain length.

**Table 7: Composition of Liposomes with Various Phospholipids**

<b>Formulation</b>	<b>Phospholipid/Cholesterol</b>	<b>Molar Ratio</b>
(M)	Distearoylphosphatidylcholine (C18)/Chol	70/30
(N)	Dipalmitoylphosphatidylcholine (C16)/Chol	70/30
(O)	Dimyristoylphosphatidylcholine (C14)/Chol	70/30

Of the three Liposome formulations tested, the DPPC/cholesterol Liposome appears to have the highest capacity to incorporate the Nucleic Acid Ligand NX232.

**EXAMPLE 11. ANALYSIS OF THE NUCLEIC ACID LIGAND-LIPOSOME  
COMPLEX BY NON-DENATURING GEL ELECTROPHORESIS.**

In this example, the incorporation of a Nucleic Acid Ligand conjugate with cholesterol into the liposomal formulation is demonstrated. The Liposome formulation used in this study is the DSPC:Cholesterol (2:1, mol/mol). The ability of the Liposome to incorporate the cholesterylated thrombin ligand NX 253, radiolabeled with <sup>125</sup>I-Bolton-Hunter reagent (5'-[Cholesterol][dT-NH-<sup>125</sup>I-Bolton-Hunter]-d(CAG TCC GTG GTA GGG CAG GTT GGG GTG ACT TCG TGG

AA)[3'3'dT]dT-3' (SEQ ID NO:8), where dT-NH-<sup>125</sup>I-Bolton-Hunter is the Amino-Modifier C6 dT (Glen Research, Sterling, VA) conjugated to the Bolton-Hunter reagent (New England Nuclear, Boston, MA) and 3'3'dT (dT-5'-CE phosphoramidite, Glen Research, Sterling, VA) is the inverted-orientation phosphoramidite) was examined by determining the fraction of bound Nucleic Acid Ligand as a function of Liposome:Nucleic Acid Ligand ratio. The Nucleic Acid Ligand-Liposome Complexes were prepared by incubating the Nucleic Acid Ligand with the Liposome in 25 mM Tris buffer, pH 7.4 containing 9% sucrose at 65 °C for 15 min. The free Nucleic Acid Ligand can be separated from the Liposome-bound Nucleic Acid Ligand by non-denaturing polyacrylamide gel electrophoresis. This method allows for rapid and complete separation of the two species. In order to allow the Liposome-bound Nucleic Acid Ligand to enter the gel (so that it can be visualized), it is necessary to disrupt the Liposomes by adding a 1% solution of triton X-100 to the loading wells for about 5 minutes prior to termination of the electrophoresis run. The amount of Liposome-bound Nucleic Acid Ligand was determined from the relative amount of the free Nucleic Acid Ligand, which runs as a well-defined band, by phosphorimager analysis. Assuming that there are approximately 60,000 lipids per Liposome, and that the mean MW of a lipid is 655.9 Da ( $= 790.15 \times 0.67 + 386.7 \times 0.33$ ), the saturation of the Liposome with the Nucleic Acid Ligand occurs at the molar ratio of Nucleic Acid Ligand to Liposome of approximately 300 (Figure 12). The analog of NX253 that does not have the cholesterol moiety is not incorporated into the Liposome over the same range of Liposome concentrations (data not shown).

**EXAMPLE 12. PASSIVE ENCAPSULATION OF NUCLEIC ACID LIGANDS INTO LIPOSOMES**

The Nucleic Acid Ligands are encapsulated within the aqueous interior of Liposomes. An aqueous solution of a Nucleic Acid Ligand is prepared by dissolving the Nucleic Acid Ligands in phosphate buffer solution (PBS) to yield a stock solution with a concentration of approximately 3.5 mg/ml. A lipid film containing DSPC:Chol (2:1 mole ratio) is prepared by drying the lipid mixture from chloroform:methanol:water (1:5:1, v:v:v) solvent. One ml of the Nucleic Acid stock solution is added to the lipid film and bath sonicated at a temperature of 40°C for 10 seconds. The resulting solution is put through a 4-cycle freeze-thaw procedure using

liquid nitrogen. The resulting homogeneous solution is extruded first through a 0.8  $\mu\text{m}$  filter membrane (3 times) then extruded through a 0.45  $\mu\text{m}$  filter (3 times) and finally through a 0.2  $\mu\text{m}$  filter (3 times). Unencapsulated Nucleic Acid Ligands are removed by passing the dispersion through a Sephadex G-50 column with a bed volume of about 20 ml.

**EXAMPLE 13. REMOTE LOADING OF NUCLEIC ACID LIGANDS INTO LIPOSOMES**

The Nucleic Acid Ligands are encapsulated within the aqueous interior of MLVs by remote loading. A lipid mixture of DSPC:Chol (2:1 mole ratio) is prepared as a lipid film using 20  $\mu\text{mol}$  of lipid. The lipid film is vortexed into suspension using 0.1 M  $\text{MgCl}_2$  at 65°C to form MLVs having an average diameter of one micron. The Liposome suspension is frozen in liquid nitrogen and thawed at 65°C. The freeze/thaw cycling is repeated three times to ensure that the salt is uniformly distributed throughout the lamellae. The osmolarity of the internal aqueous phase is approximately 300 milliosmoles (mOsm). The Liposome suspension is pelleted by centrifugation at 10,000 g for 15 minutes to remove external  $\text{MgCl}_2$  solution. The supernatant is removed and the Liposome pellet is heated at 65°C for 5 minutes. A solution of Nucleic Acid Ligand (20  $\mu\text{g}$  in 100  $\mu\text{l}$  water) is preheated for 5 minutes at 65°C and added to the Liposome pellet. Heating is continued for 30 minutes and the sample is then slowly cooled to room temperature and diluted with 1 ml PBS. Untrapped Nucleic Acid Ligand is removed by centrifugation of the MLVs followed by supernatant removal. The pellet is resuspended in fresh PBS and re-pelleted by centrifugation.

**EXAMPLE 14. COVALENT CONJUGATION OF NUCLEIC ACID LIGANDS TO LIPOSOMES**

In scheme 1 provided below, a heterobifunctional PEG-2000 (PEG with molecular weight 2000 Da) containing a N-hydroxysuccinimide ester and vinyl sulfone functionalities was first conjugated to a Liposome containing 2 mole % distearylphosphatidylethanolamine (DSPE) via the N-hydroxysuccinimide ester moiety. The product was purified from the free PEG by size exclusion



chromatography. The vinyl sulfone product was then allowed to react with reduced NX 256 (Figure 1D; SEQ ID NO:9). A DSPE-PEG-2000-vinyl sulfone is commercially available and can be used to manufacture Liposomes that contain the vinyl sulfone functionality, thus eliminating a conjugation step from Scheme 1.

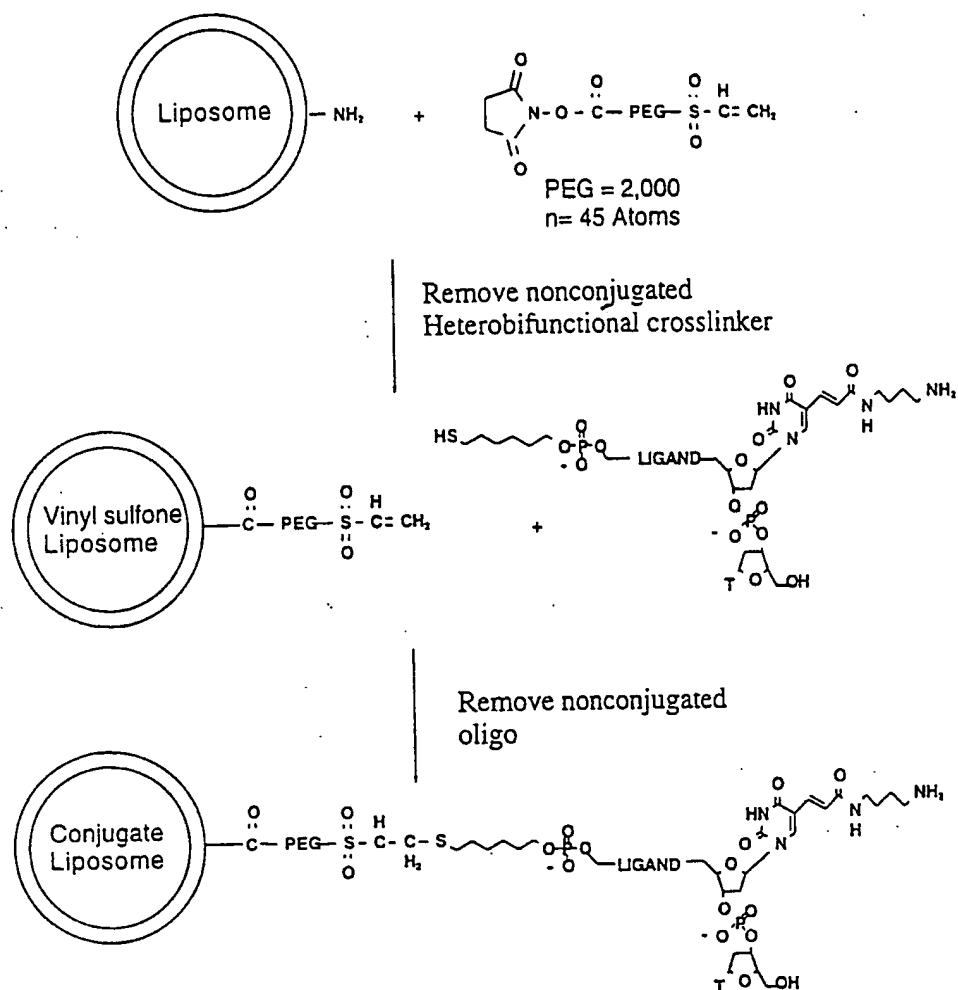
5

10

15

20

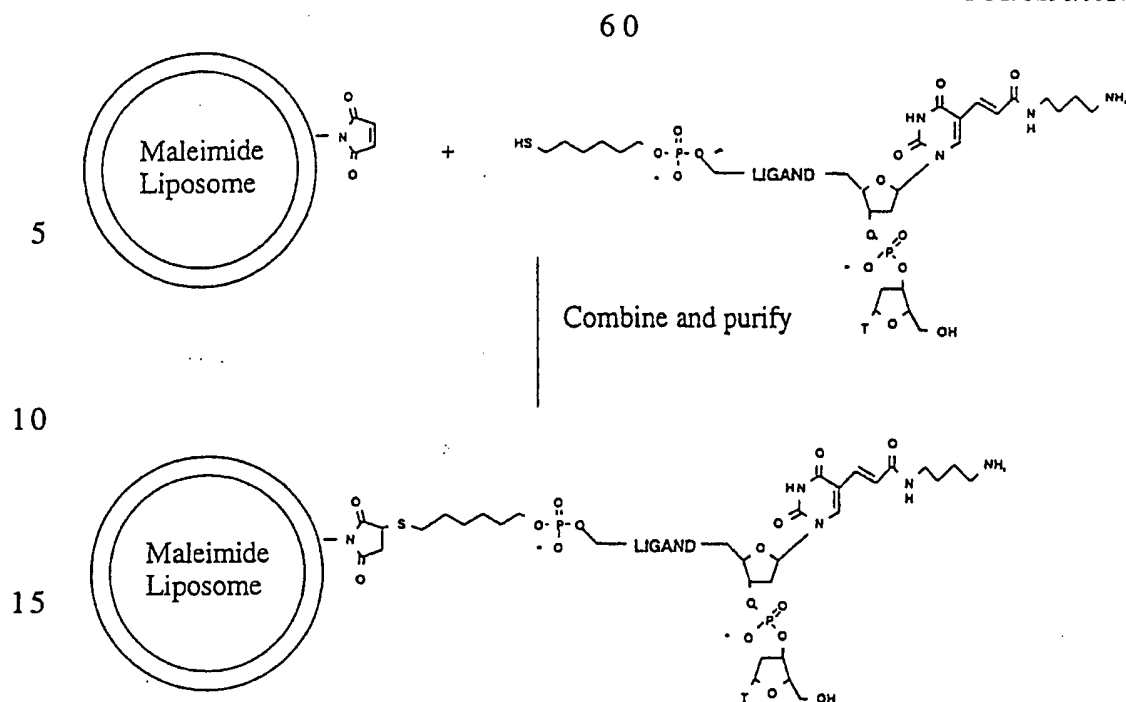
25



30

The second reaction, shown below as scheme 2, has been completed. The starting material was a distearylphosphatidylcholine (DSPC) Liposome containing 2 mole % DSPE maleimide. Using a value of 50,000 lipids per Liposome, the Liposome should have approximately 1000 maleimide molecules per Liposome, about 600 of which are available on the outside. The Nucleic Acid Ligand-Liposome Complex was separated from the free Nucleic Acid Ligand via size exclusion chromatography (*vide supra*). From the absorbance at 260 nm, it was estimated that approximately 200 molecules of the Nucleic Acid Ligand were conjugated to each Liposome.

35



20 **EXAMPLE 15. IN VITRO AND IN VIVO EFFICACY OF NUCLEIC ACID**  
**LIGAND-LIPOSOME COMPLEX. DIALKYLGLYCEROL (DAG)-MODIFIED**  
**VEGF LIGAND (NX278) EMBEDDED IN LIPOSOME BILAYER.**

NX278-Liposome Complex was prepared by incubating NX-278 (1 mg) (Figure 1N; SEQ ID NO:19) with of a mixture of DSPC:cholesterol (50 mg) in 10 mM phosphate (pH 7.4) buffer containing 9% sucrose and sonicated for 15-30 min using a probe-type sonicator until opalescent solution was obtained. The control Nucleic Acid Ligand-Liposome Complex containing a sequence scrambled analog of ligand NX-278 (scNX278) (Figure 1W; SEQ ID NO:28) was prepared in the same manner. The size of Liposome particles (typically 50-100 nM), determined in a particle analyzer (Leeds & Northrup Model Microtrack UPA 150, Horsham, PA) was similar to those obtained in the absence of the Nucleic Acid Ligand. NX278-Liposome Complex competed with a biotin-labeled Nucleic Acid Ligand to VEGF for binding to polystyrene-immobilized VEGF in a competition ELISA assay with an apparent ED50 of  $\approx 10^{-7}$  M. In the same assay, scNX278-Liposome Complex was not an effective competitor up to 2  $\mu$ M Nucleic Acid Ligand. For comparison, free Nucleic Acid Ligand to VEGF with the same sequence as NX278 but lacking the DAG moiety at the 5' end, NX213 (Figure 1P; SEQ ID NO:21), exhibited a competition ED50 value of  $\approx 10^{-9}$  M. The reduced ability of NX278-Liposome compared to NX213 to bind to immobilized VEGF may

be due to a simple geometric constraint, since only a fraction of the Nucleic Acid Ligand displayed on the outer surface of the Liposomes is expected to be available for binding to a planar surface. In addition, the fraction of Nucleic Acid Ligand displayed on the inner surface would obviously not be available for binding in this assay.

5           The effects of NX278-liposome, scNX278-liposome and NX213 on the proliferation of human umbilical vein endothelial cells (HUVEC) and Kaposi's Sarcoma (KS) cells in tissue culture were examined. HUVECs were grown in the presence of VEGF (10 ng/ml) in IMDM:Ham's F12 (1:1) medium containing 10% fetal calf serum (FCS) and heparin (45 µg/ml). Cells were plated in 24-well gelatin-coated plates at a  
10           density of 20,000 cells per well on day zero and treated with the above ligands at concentrations between 0.1 nM to 1 µM on days 1, 2, and 3 (replacing the media along with the ligands). Cell count was performed on day 4. KS cell line KSY-1 was plated in 24-well gelatin coated plates at a density of 7,500-10,000 cells per well on day zero in medium containing RPMI 1640 supplemented with 2% FCS, L-glutamine, penicillin  
15           and streptomycin. Nucleic Acid Ligands were added at concentrations between 0.1 nM to 1 µM in fresh medium on day 1, 2, and 3 and the cell count was performed on day 4. NX278-Liposome inhibited the proliferation of HUVECs with an IC<sub>50</sub> of ≈300 nM (the concentration refers to the Nucleic Acid Ligand component); the free Nucleic Acid analog, NX213, was significantly less effective (IC<sub>50</sub> >1 µM). NX278-  
20           Liposome also inhibited the proliferation of KS cells with an IC<sub>50</sub> of ≈100 nM; at 1 µM NX278-Liposome, the growth of these cells was completely inhibited. scNX278-Liposome and NX213 exhibited IC<sub>50</sub> values of >1 µM.

          The ability of NX278-liposome to inhibit the vascular permeability activity of VEGF *in vivo* was examined. The vascular permeability assay (also known as the  
25           Miles assay (Miles, A. A. and Miles, E. M. (1952) J. Physiol. (London) 118:228) was performed in guinea pigs essentially as described (Senger, R. S. *et al.*, (1983) Science 219:983). NX278-Liposome at the concentration of 1 µM significantly inhibited the VEGF-induced vascular permeability increase. The control compound, scNX278-Liposome was not inhibitory at this concentration; in fact the vascular permeability  
30           appeared to be enhanced.

**WE CLAIM:**

1. A therapeutic or diagnostic Complex comprised of a Nucleic Acid Ligand and a Lipophilic Compound or a Non-Immunogenic, High Molecular Weight Compound.

2. The Complex of claim 1 wherein said Nucleic Acid Ligand was identified as a ligand of a given Target from a Candidate Mixture of Nucleic Acids according to the method comprising:

a) contacting the Candidate Mixture with the Target, wherein the Nucleic Acids having an increased affinity to the Target relative to the Candidate Mixture may be partitioned from the remainder of the Candidate Mixture;

b) partitioning the increased affinity Nucleic Acids from the remainder of the Candidate Mixture; and

c) amplifying the increased affinity Nucleic Acids to yield a ligand-enriched mixture of Nucleic Acids.

3. The Complex of claim 2 wherein said method further comprises:

d) repeating steps b) and c).

4. The Complex of claim 1 comprised of a Nucleic Acid Ligand and a Non-Immunogenic, High Molecular Weight Compound.

5. The Complex of claim 4 wherein said Non-Immunogenic, High Molecular Weight Compound is selected from the group consisting of polyethylene glycol, dextran, albumin, and magnetite.

6. The Complex of claim 4 wherein said Non-Immunogenic, High Molecular Weight Compound is polyethylene glycol.

7. The Complex of claim 1 comprised of a Nucleic Acid Ligand and a Lipophilic Compound.

8. The Complex of claim 7 wherein said Lipophilic Compound is selected from the group consisting of cholesterol, dialkyl glycerol, and diacyl glycerol.
9. The Complex of claim 8 wherein said Lipophilic Compound is cholesterol.
10. The Complex of claim 8 wherein said Lipophilic Compound is dialkyl glycerol.
11. The Complex of claim 9 further comprising a Lipid Construct.
12. The Complex of claim 11 wherein said Lipid Construct is a Lipid Bilayer Vesicle.
13. The Complex of claim 12 wherein said Lipid Bilayer Vesicle is a Liposome.
14. The Complex of claim 7 further comprising a Lipid Construct.
15. The Complex of claim 14 wherein said Lipid Construct is a Lipid Bilayer Vesicle.
16. The Complex of claim 15 wherein said Lipid Bilayer Vesicle is a Liposome.
17. The Complex of claim 10 further comprising a Lipid Construct.
18. The Complex of claim 17 wherein said Lipid Construct is a Lipid Bilayer Vesicle.
19. The Complex of claim 18 wherein said Lipid Construct is a Liposome.
20. The Complex of claim 6 further comprising a Lipid Construct.

21. The Complex of claim 20 wherein said Lipid Construct is a Lipid Bilayer Vesicle.

22. The Complex of claim 21 wherein said Lipid Bilayer Vesicle is a Liposome.

23. The Complex of claim 16 wherein said Nucleic Acid Ligand is encapsulated within the interior of said Liposome.

24. The Complex of claim 16 wherein said Nucleic Acid Ligand is non-covalently associated with the exterior of said Liposome.

25. The Complex of claim 16 wherein said Nucleic Acid Ligand is covalently attached to a second Lipophilic Compound.

26. The Complex of claim 25 wherein said Nucleic Acid Ligand is attached to a second Lipophilic Compound via a Linker.

27. The Complex of claim 25 wherein said Nucleic Acid Ligand projects out of the exterior surface of said Liposome.

28. The Complex of claim 25 wherein said Nucleic Acid Ligand projects into the interior of said Liposome.

29. The Complex of claim 16 further comprising a therapeutic or diagnostic agent.

30. The Complex of claim 29 wherein said therapeutic agent is encapsulated in the interior of said Liposome.

31. The Complex of claim 29 wherein said therapeutic or diagnostic agent is associated with the membrane of said Liposome.

32. The Complex of claim 29 wherein said drug or diagnostic agent is associated with the exterior surface of said Liposome.
33. The Complex of claim 1 wherein said Nucleic Acid Ligand targets the Complex to a preselected location.
34. The Complex of claim 1 wherein the pharmacokinetic properties of said Complex are improved relative to the Nucleic Acid Ligand alone.
35. The Complex of claim 1 wherein the SELEX Target of said Nucleic Acid Ligand is an intercellular SELEX Target.
36. The Complex of claim 1 wherein the SELEX Target of said Nucleic Acid Ligand is an intracellular SELEX Target.
37. The Complex of claim 36 wherein the cellular uptake of said Nucleic Acid Ligand is enhanced relative to the Nucleic Acid Ligand alone.
38. The Complex of claim 6 further comprising a therapeutic or diagnostic agent.
39. The Complex of claim 38 wherein said therapeutic or diagnostic agent is associated with said polyethylene glycol.
40. The Complex of claim 39 wherein said therapeutic or diagnostic agent is covalently associated with said polyethylene glycol.
41. The Complex of claim 1 wherein said Nucleic Acid Ligand is covalently bound to said Lipophilic Compound or said Non-Immunogenic, High Molecular Weight Compound.

42. The Complex of claim 1 wherein said Nucleic Acid Ligand is noncovalently associated with said Lipophilic Compound or said Non-Immunogenic, High Molecular Weight Compound.

43. A method for the preparation of a therapeutic or diagnostic Complex comprised of a Nucleic Acid Ligand and a Lipophilic Compound or a Non-Immunogenic, High Molecular Weight Compound, said method comprising:

identifying a Nucleic Acid Ligand from a Candidate Mixture of Nucleic Acids, said Nucleic Acid Ligand being a ligand of a given Target by the method comprising:

a) contacting the Candidate Mixture with the Target, wherein Nucleic Acids having an increased affinity to the Target relative to the Candidate Mixture may be partitioned from the remainder of the Candidate Mixture;

b) partitioning the increased affinity Nucleic Acids from the remainder of the Candidate Mixture; and

c) amplifying the increased affinity Nucleic Acids to yield a ligand-enriched mixture of Nucleic Acids; and

d) associating said identified Nucleic Acid Ligand with a Lipophilic Compound or a Non-Immunogenic, High Molecular Weight Compound.

44. The method of claim 43 wherein said Complex is comprised of a Nucleic Acid Ligand and Non-Immunogenic, High Molecular Weight Compound.

45. The method of claim 44 wherein said Non-Immunogenic, High Molecular Weight Compound is selected from the group consisting of polyethylene glycol, dextran, albumin, and magnetite.

46. The method of claim 45 wherein said Non-Immunogenic, High Molecular Weight Compound is polyethylene glycol.

47. The method of claim 43 wherein said Complex is comprised of a Nucleic Acid Ligand and Lipophilic Compound.



48. The method of claim 47 wherein said Lipophilic Compound is selected from the group consisting of cholesterol, dialkyl glycerol and diacyl glycerol.

49. The method of claim 48 wherein said Lipophilic Compound is cholesterol.

50. The method of claim 48 wherein said Lipophilic Compound is dialkyl glycerol.

51. The method of claim 49 wherein said Complex further comprises a Lipid Construct.

52. The method of claim 51 wherein the Lipid Construct is a Lipid Bilayer Vesicle.

53. The method of claim 52 wherein said Lipid Bilayer Vesicle is a Liposome.

54. The method of claim 50 wherein said Complex further comprises a Lipid Construct.

55. The method of claim 54 wherein said Complex further comprises a Lipid Bilayer Vesicle.

56. The method of claim 55 wherein said Lipid Bilayer Vesicle is a Liposome.

57. The method of claim 56 wherein said Nucleic Acid Ligand is projecting from the exterior surface of said Liposome.

58. The method of claim 47 wherein said Complex further comprises a Lipid Construct.

59. The method of claim 58 wherein said Lipid Construct is a Lipid Bilayer vesicle.

60. The method of claim 59 wherein said Lipid Bilayer Vesicle is a Liposome.

61. The method of claim 60 wherein said Nucleic Acid Ligand is projecting from the exterior surface of said Liposome.

The method of claim 60 wherein said Complex further comprises a therapeutic or diagnostic agent.

63. The method of claim 43 wherein said Nucleic Acid Ligand targets the Complex to a preselected location.

64. The method of claim 43 wherein the Pharmacokinetic Properties of said Complex are improved relative to the Nucleic Acid Ligand alone.

65. The method of claim 46 wherein said Complex further comprises a therapeutic or diagnostic agent.

66. The method of claim 65 wherein said therapeutic or diagnostic agent is associated with said polyethylene glycol.

67. A method for improving the pharmacokinetic properties of a Nucleic Acid Ligand comprising:

associating said Nucleic Acid Ligand to a Lipophilic Compound or a Non-Immunogenic, High Molecular Weight Compound to form a Complex comprised of a Nucleic Acid Ligand and a Lipophilic Compound or a Non-Immunogenic, High Molecular Weight Compound; and administering said Complex to a patient.

68. A method for improving the cellular uptake of a Nucleic Acid Ligand having an intracellular SELEX Target comprising:

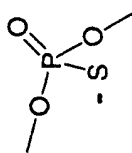
associating said Nucleic Acid Ligand with a Lipophilic Compound or a Non-Immunogenic, High Molecular Weight Compound to form a Complex comprising of a Nucleic Acid Ligand and a Lipophilic Compound or a Non-Immunogenic, High Molecular Weight Compound; and administering said Complex to a patient.

69. A method for targeting a therapeutic or diagnostic agent to a specific predetermined biological Target in a patient comprising:

associating said therapeutic or diagnostic agent with a Complex comprised of a Nucleic Acid Ligand and a Lipophilic Compound or a Non-Immunogenic, High Molecular Weight Compound; wherein said Nucleic Acid Ligand has a SELEX Target associated with said specific predetermined biological Target, and said Nucleic Acid Ligand is associated with the exterior of the Complex; and administering said Complex to a patient.

NX-229  
SEQ ID NO: 6

Ligand = 5'-CAGTCCGTGGTAGGGCAGGTTGGGGTGACTTCGTGGAs-3'  
(Thrombin ligand)



s = Phosphorothioate =

1/34

FIGURE 1A

2/34

NX-232  
SEQ ID NO: 7

Structure =

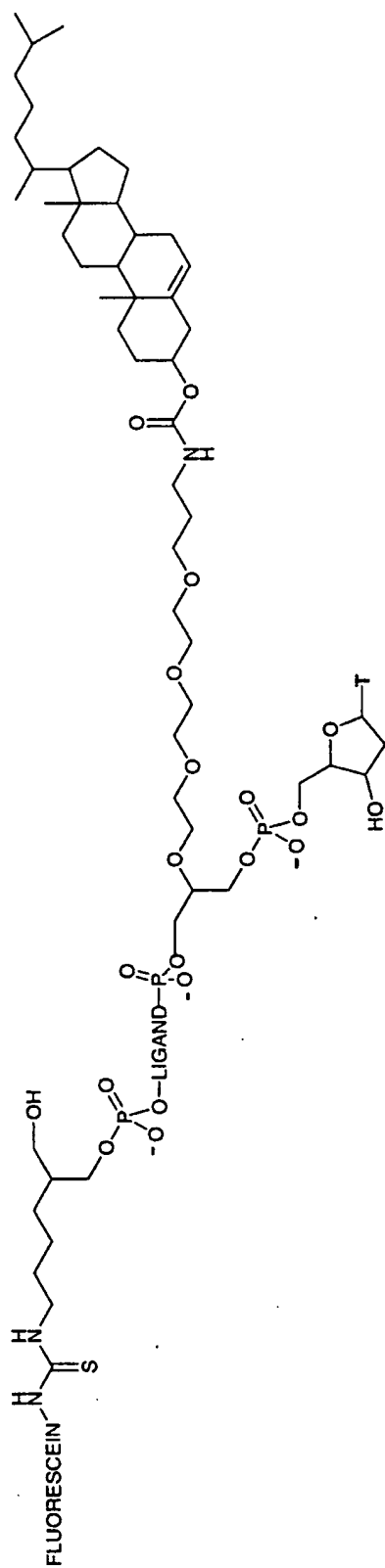
Ligand Component = 5'-CAGTCCGTGGTAGGGCAGGTTGGGGTGACTTCGTGGAA-3'  
(Thrombin ligand)

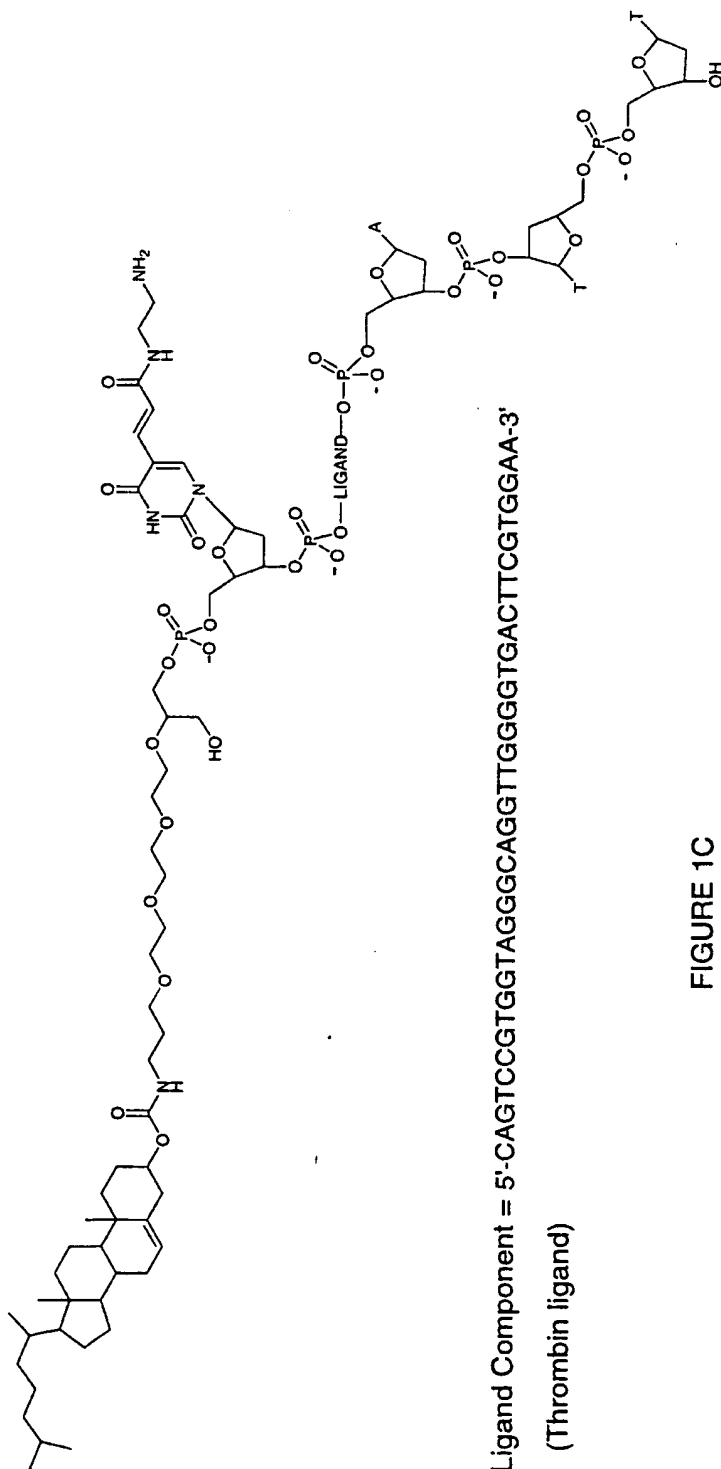
FIGURE 1B

3/34

NX-253

SEQ ID NO: 8

Structure =



Ligand Component = 5'-CAGTCCGTGGTAGGGCAGGTTGGGGTGACTTCGTGGAA-3'  
(Thrombin ligand)

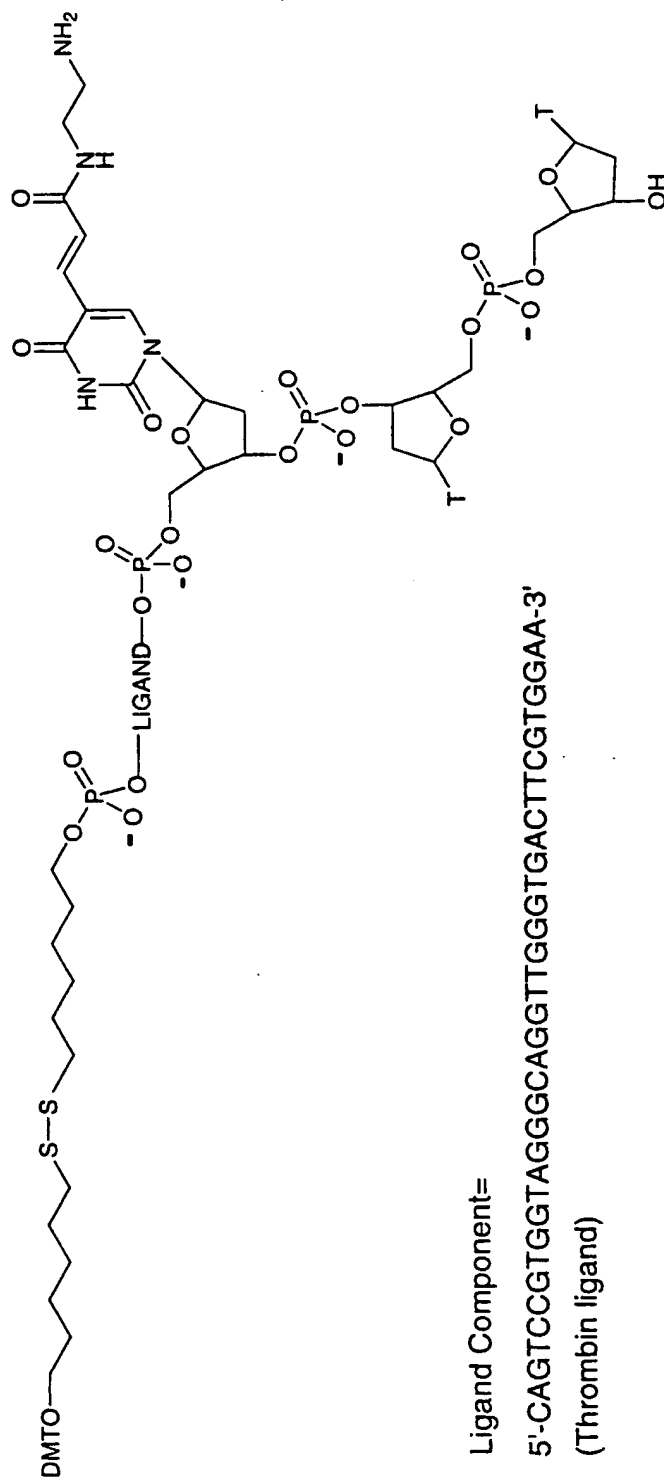
FIGURE 1C

4/34

NX-256

SEQ ID NO: 9

Structure =



Ligand Component=

5'-CAGTCCGTGGTAGGGCAGGTTGGGTGACTTCGTGGAA-3'

(Thrombin ligand)

FIGURE 1D

5/34

225T3  
SEQ ID NO: 10

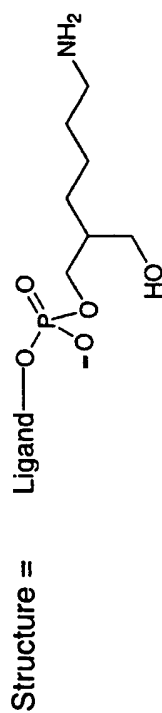


FIGURE 1E

Ligand component = 5'-GCGGGGCTACGTACCGGGGCTTTGTAAACCCCGC-3'  
(bFGF ligand)

225T3N  
SEQ ID NO: 11

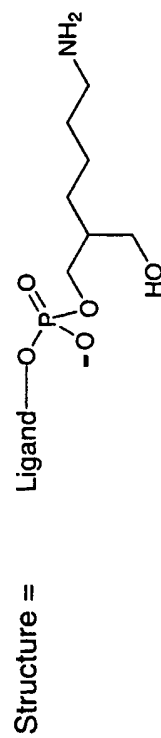


FIGURE 1F

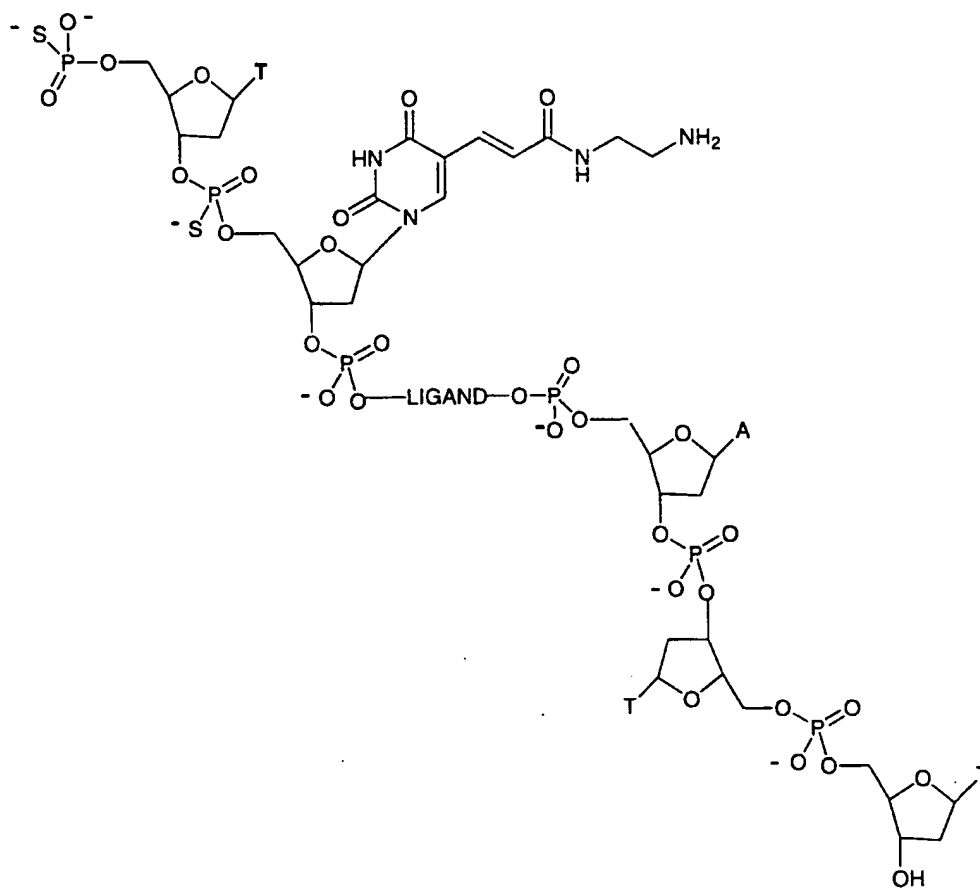
Ligand component = 5' - GCGGGGCTACGTACCGGGGCTTTGTAAACCCCGC-3'  
(bFGF ligand)



6/34

T-P4  
SEQ ID NO: 12

Structure =



Ligand component =

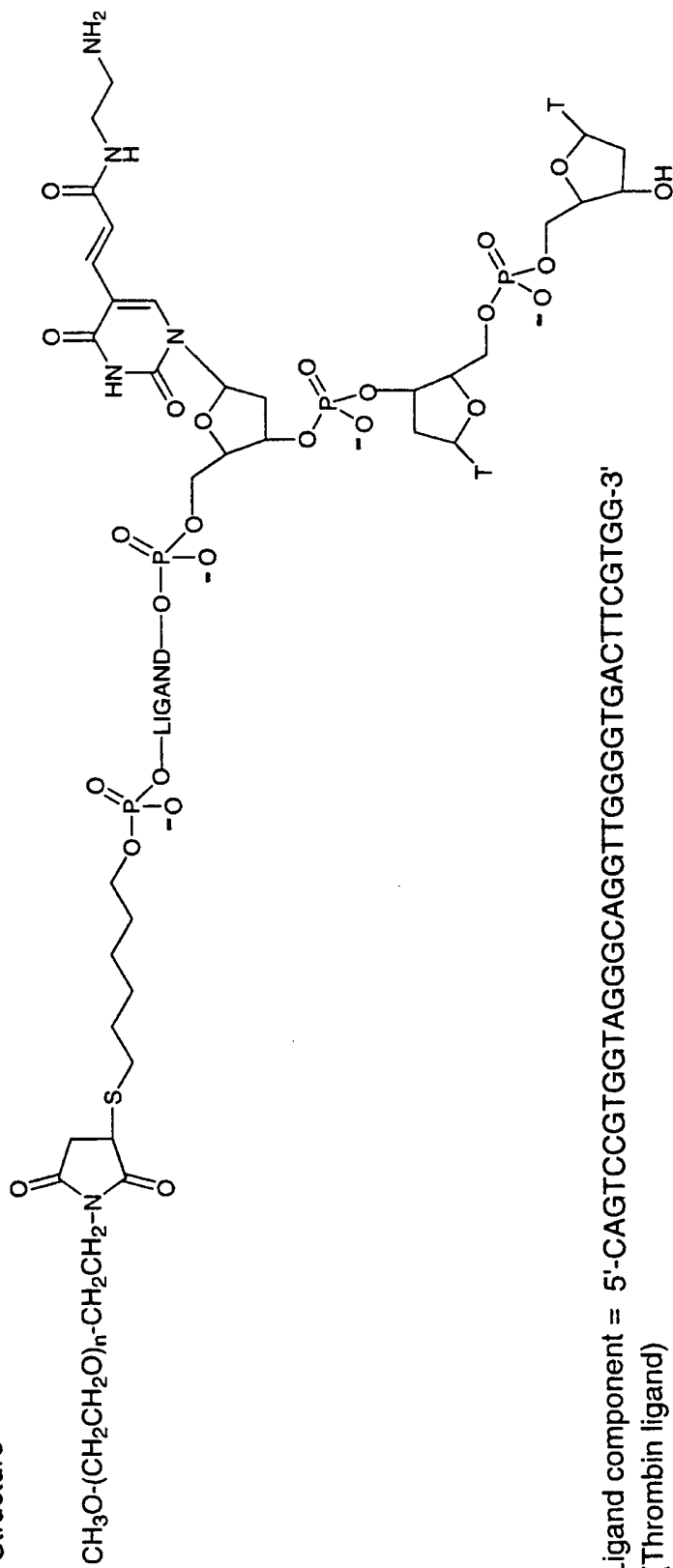
5'-CAGTCCGTGGTAGGGCAGGTTGGGGTGACTTCGTGGA-3'  
(Thrombin ligand)

FIGURE 1G

7/34

NX-256-PEG-20,000  
SEQ ID NO: 13

Structure =



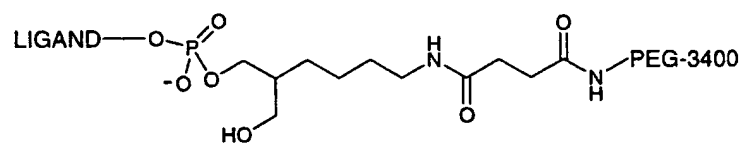
Ligand component = 5'-CAGTCCGTGGTAGGGCAGGTTGGGGTGACTTCGTGG-3'  
(Thrombin ligand)

FIGURE 1H

8/34

225T3N-PEG-3400  
SEQ ID NO: 14

Structure =



Ligand component =

5'-GCGGGGCTACGTACCGGGGCTTTGTAAAACCCCGC-3'

(bFGF ligand)

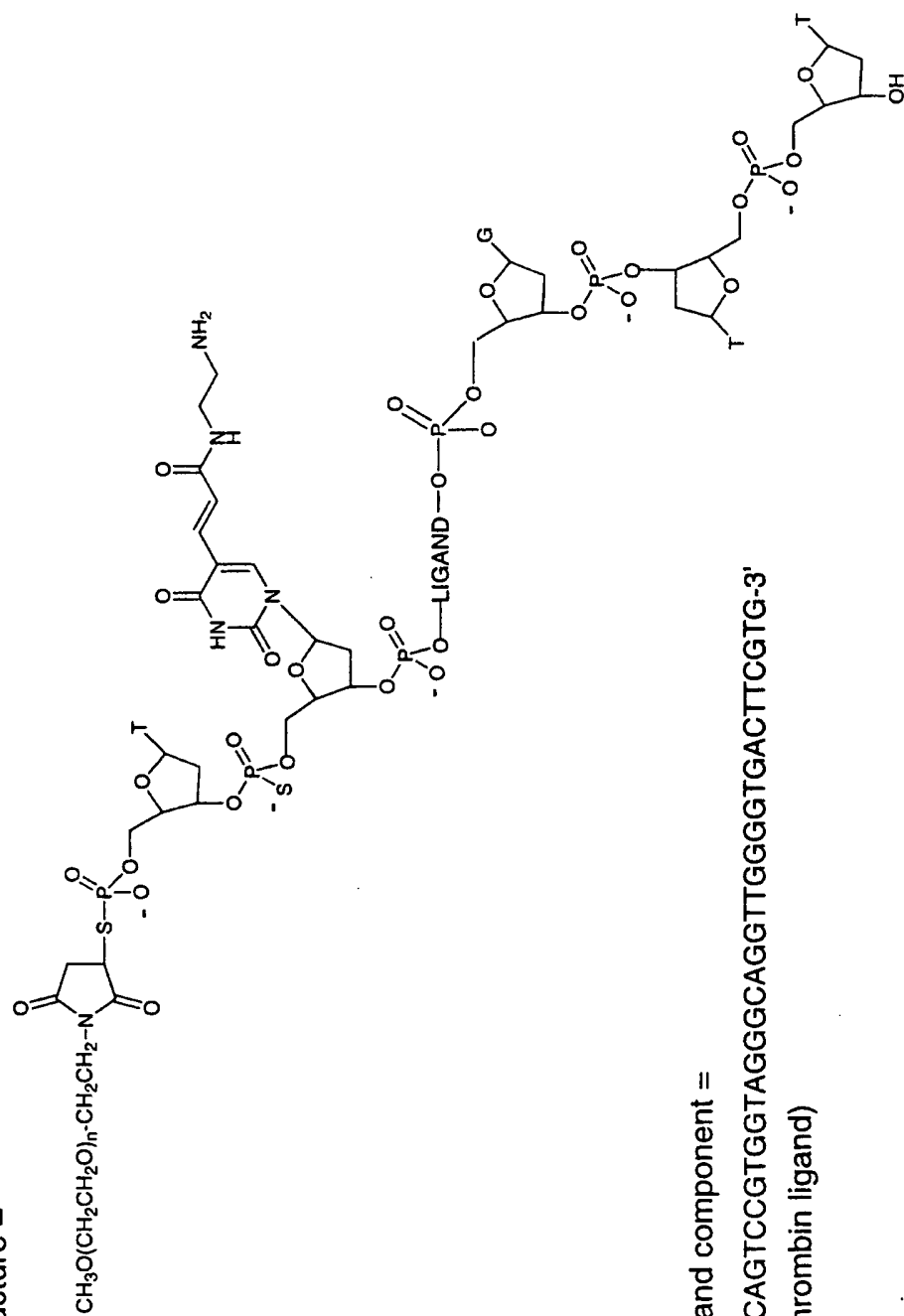
FIGURE 11

9/34

T-P4-PEG-(20,000 or 10,000)

**SEQ ID NO: 15**

**Structure =**



Ligand component =

5'-CAGTCCGTGGTAGGGCAGTTGGGGTGACTTCGTG-3'  
(Thrombin ligand)

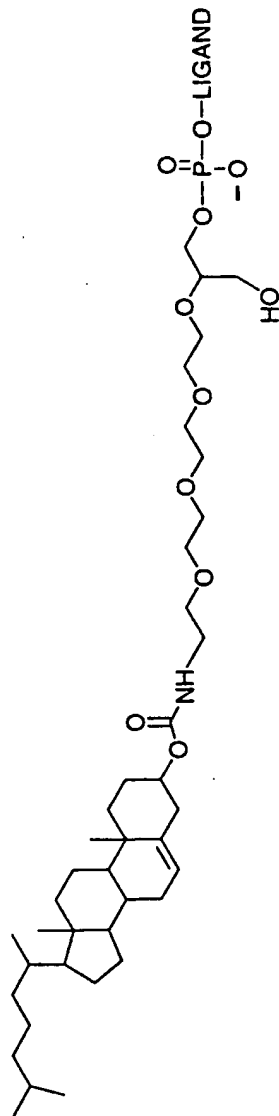
(Thrombin ligand)

FIGURE 1J

10/34

NX-268  
SEQ ID NO: 16

Structure =



Ligand component =

5'-TsTsTsmAaCaCaUrGrAaUmGrGaUmAmGrAaCmGaCaCmGmGmGaUmGTsTsTsT-3'  
(VEGF ligand)

FIGURE 1K

11/34

NX 191  
SEQ ID NO: 17

Ligand =

5'-TsTsTsTsmAmCmCmUmGmAmUmGmGmUmAmGmAmCmGmCmGmGmGmUmGTsTsTsT-3'  
(derived from VEGF ligand)

FIGURE 1L

12/34

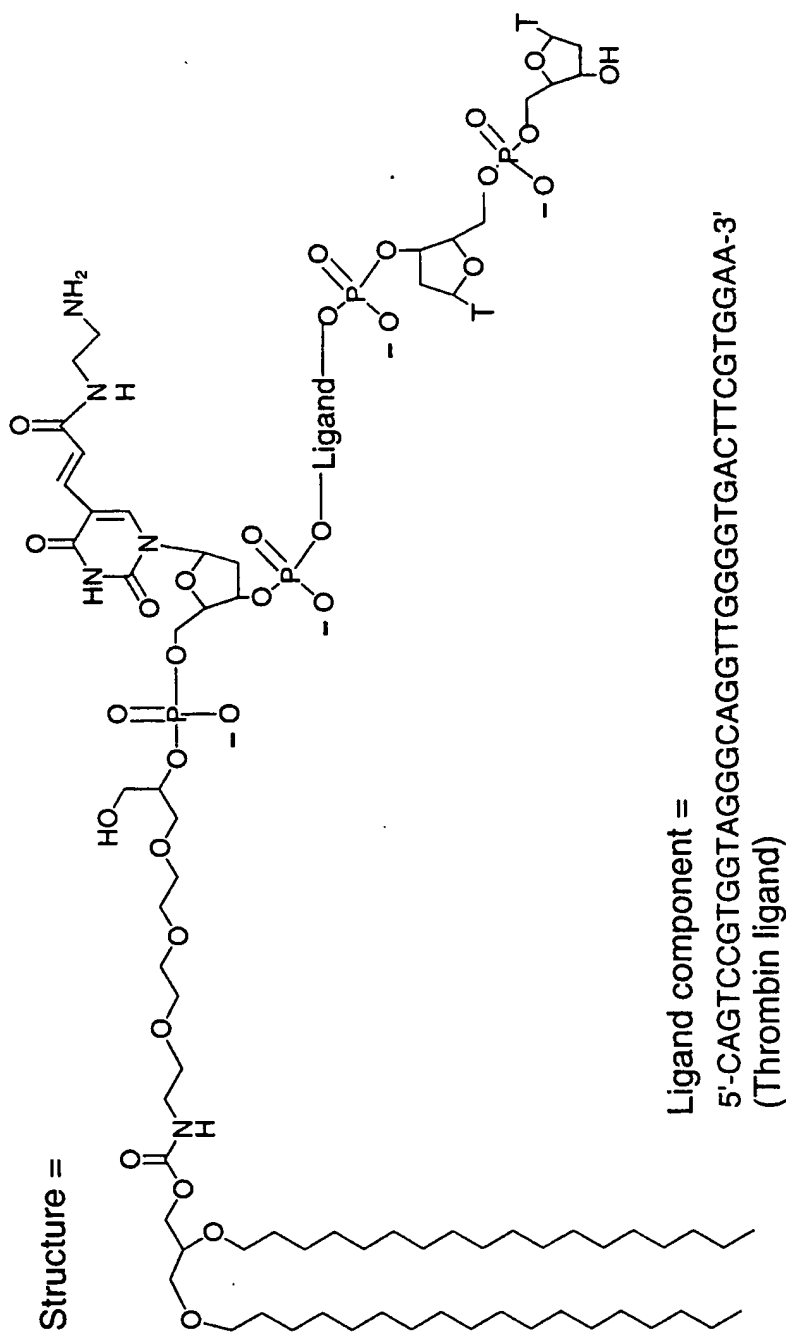
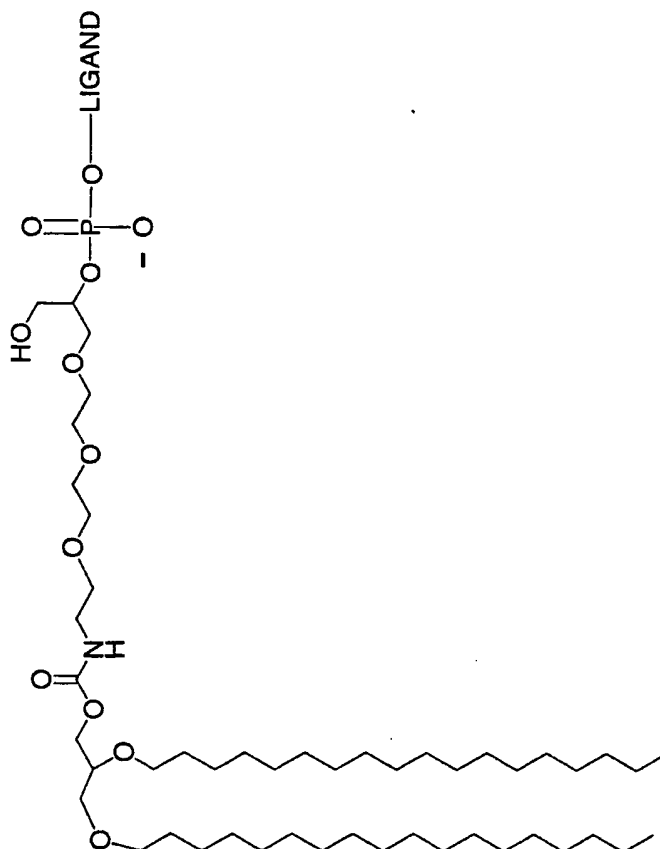
JW-966  
SEQ ID NO: 18

FIGURE 1M

NX-278

SEQ ID NO: 19

**Structure =**



**Ligand component =**

5'-TsTsTsTsmAaCaCaCaUrGrAaUmGrGaUmAmGrAaCmGaCmGmGmGmGTsTsTsTsT-3'

(VEGF ligand)

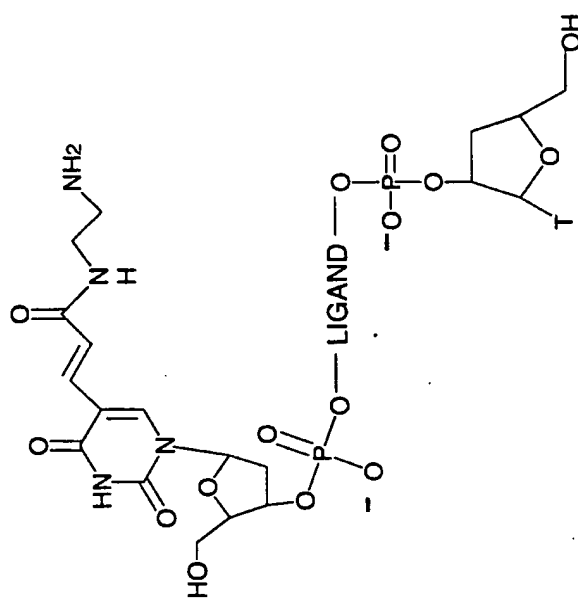
**FIGURE 1N**



14/34

JW-986  
SEQ ID NO: 20

Structure =



Ligand component =  
5'-mUmUmUmAmCmCmUmGmAmUmGmGmUmAmGmAmCmCmGmGmUmG-3'  
(derived from VEGF ligand)

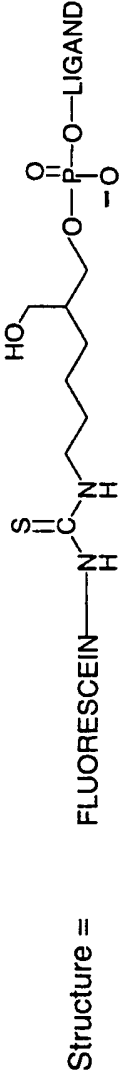
FIGURE 1 O

NX-213  
SEQ ID NO: 21

Ligand =  
5'-TsTsTsTsmAaCaCaUrGrAaUmGrGaUmAmGrAaCmGaCaCmGmGmGaUmGTsTsTsT-3'

FIGURE 1P

NX-244  
SEQ ID NO: 22



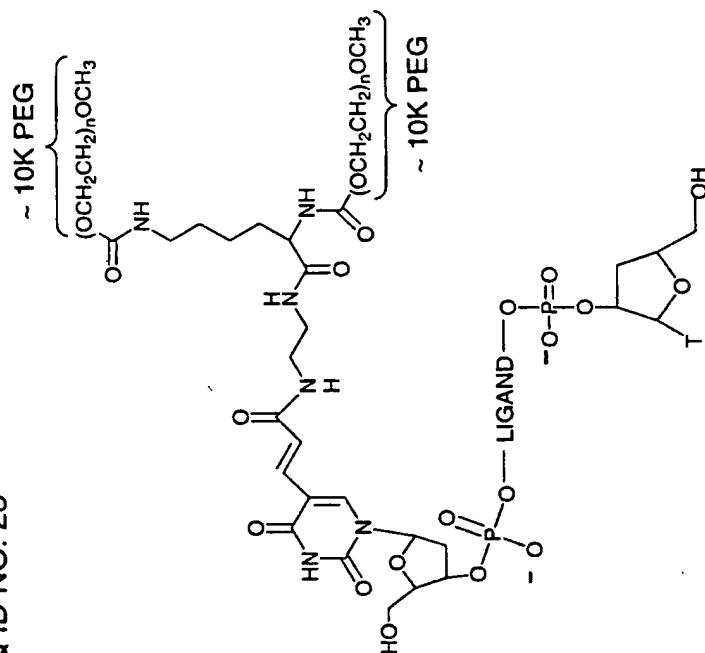
Ligand component =  
5'-TsTsTsTsmAaCaCaUrGrAaUmGrGaUmAmGrAaCmGaCaCmGmGmGaUmGTsTsTsT-3'  
(VEGF ligand)

FIGURE 1Q

16/34

**JW-1130**

SEQ ID NO: 23

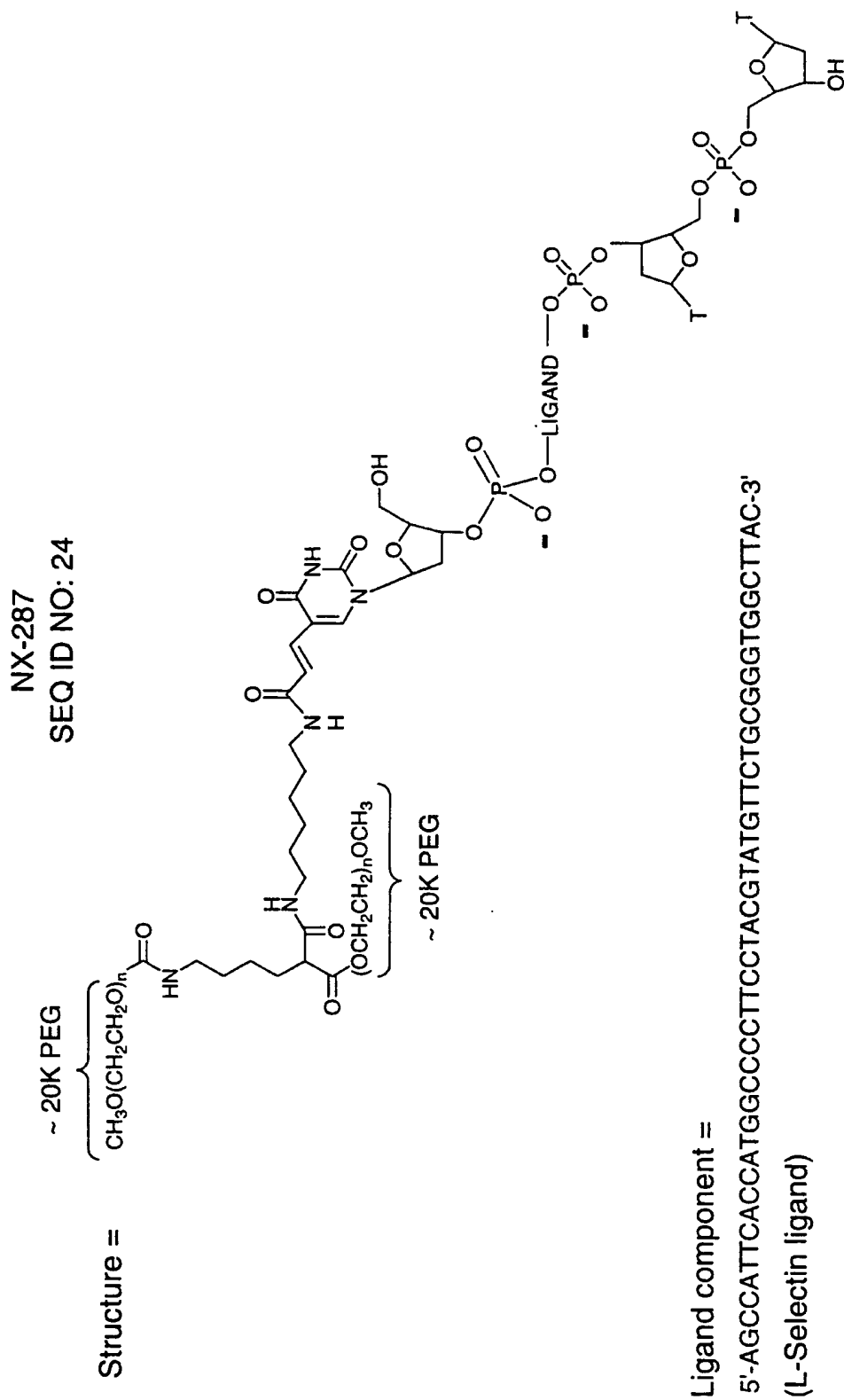


Ligand component =

5'-mUmUmUmUmAfCfCfCfUmGmAfUmGmGfUmAmGmAfCmGfCfCmGmGmGmGfUmG-3'  
(derived from VEGF ligand)

**FIGURE 1R.**

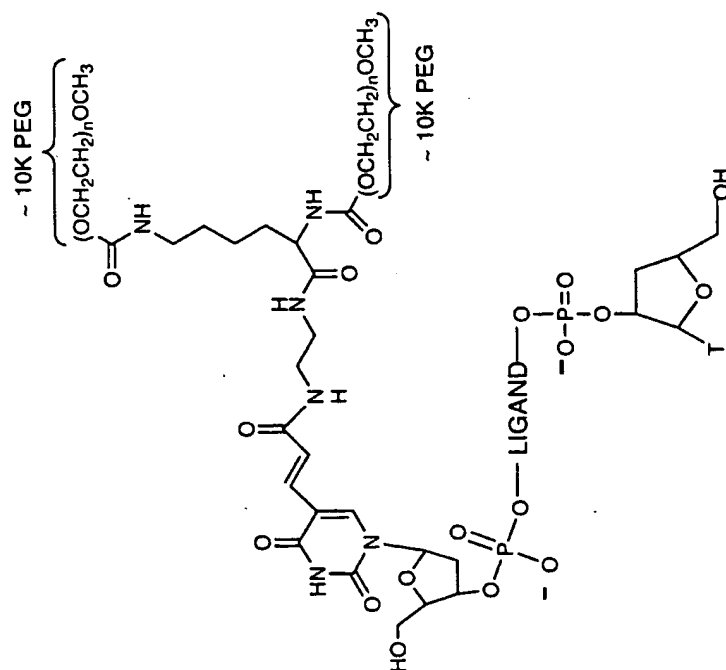
17/34



**FIGURE 1S**

18/34

JW-1336-20K PEG  
SEQ ID NO: 25



Structure =

Ligand component =

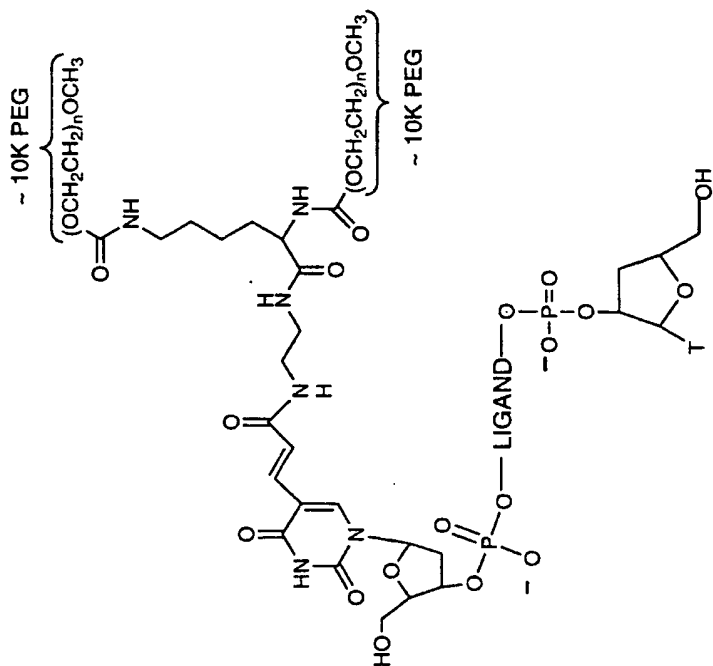
5'-mUmUmUmUAfCfCfUGAfUmGGfUmAmGAfCmGfCmGmGmGfUmG-3'  
(derived from VEGF ligand)

FIGURE 1T

19/34

JW-1379  
SEQ ID NO: 26

Structure =



Ligand component =  
5'-mUmUmUmUaFcFcFcUgGfUgAfCgFcCgGgGfUG-3'  
(derived from VEGF ligand)

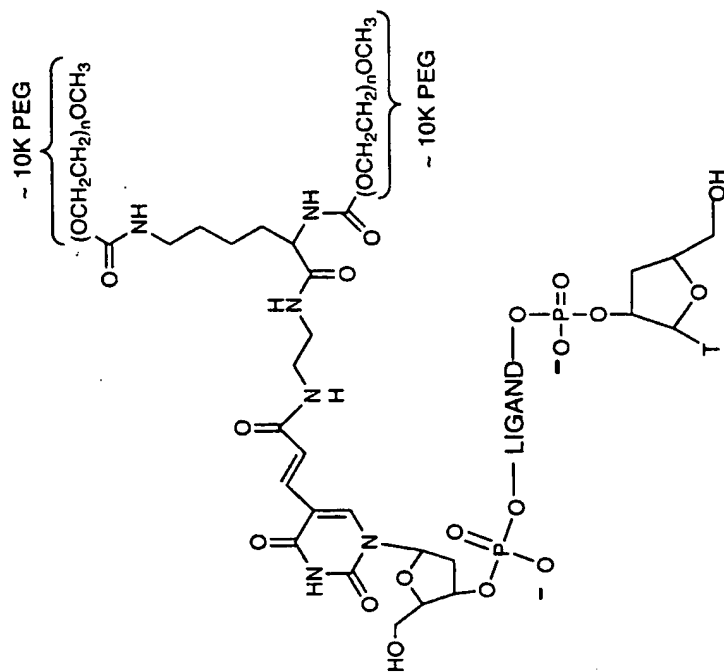
FIGURE 1U

20/34

JW-1380

SEQ ID NO: 27

Structure =



Ligand component =

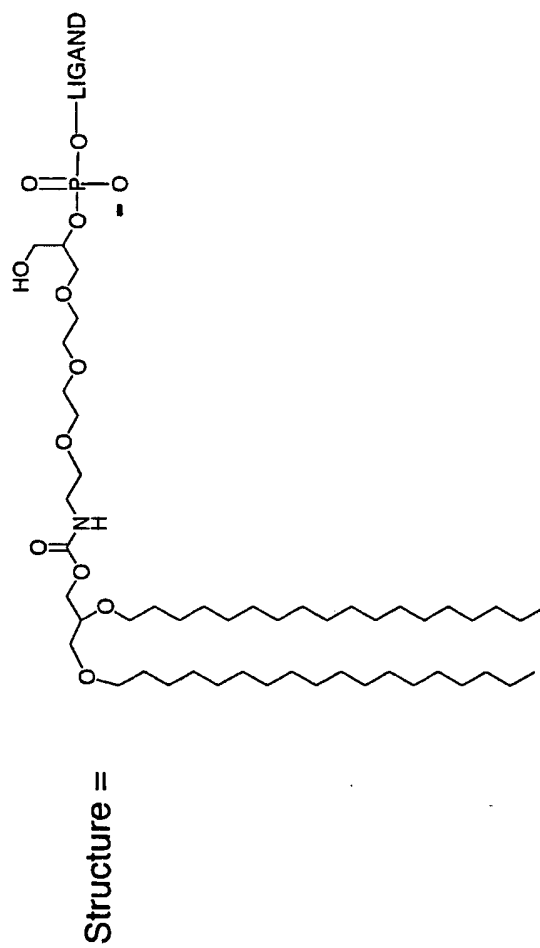
5'-mUmUmUmUACCCfUGAfUGGfUAGAfCGfCfCGGGfUGT-3'  
(derived from VEGF ligand)

FIGURE 1V

21/34

scNX-278

SEQ ID NO: 28



Ligand component =

5'-TsTsTsTs mGaUaC mGmGaU mAaCrG mGrAmG aUmGrG rAaCaC mGaUaC mAaCmG TsTsTsTsT-3'  
(VEGF ligand)

FIGURE 1W

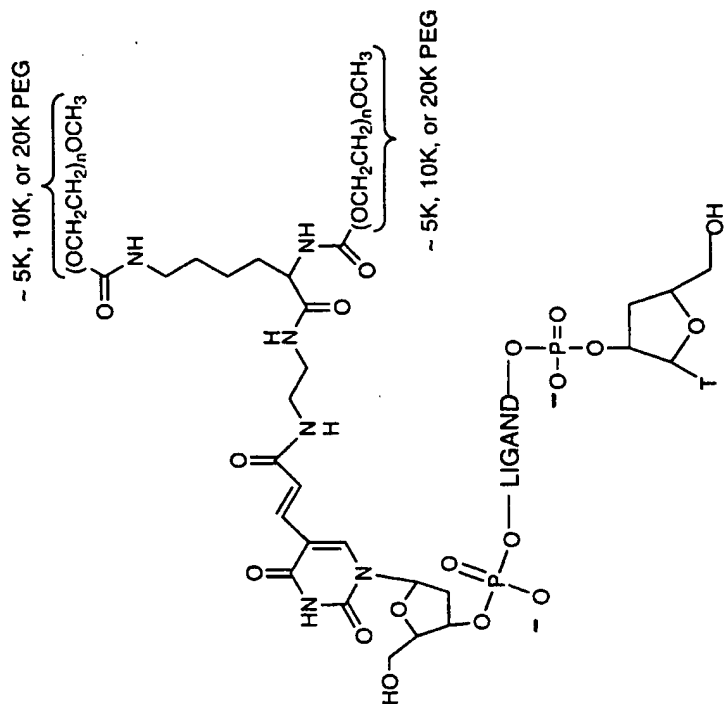


22/34

JW-986-PEG- (10,000, 20,000 or 40,000)

SEQ ID NO: 29

Structure =



Ligand component =

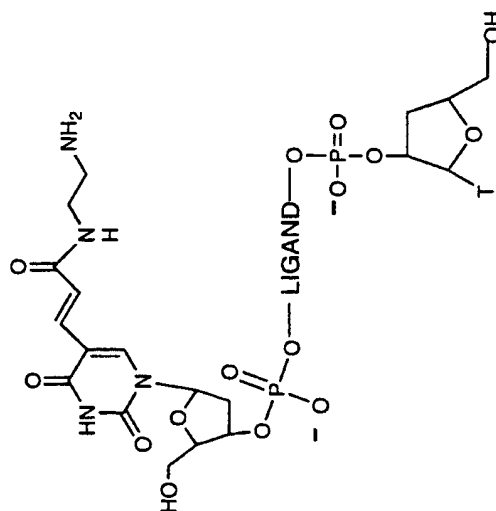
5'-mUmUmUAfCfCfUGAfUmGGfUmAmGAfCmGfCfCmGmGmGfUmG-3'  
(derived from VEGF ligand)

FIGURE 1X

23/34

JW-1336  
SEQ ID NO: 30

Structure =



Ligand component =

5'-mUmUmUAfCfCfUGAfUmGGfUmAmGAfCmGfCfCmGmGfUmG-3'

FIGURE 1Y

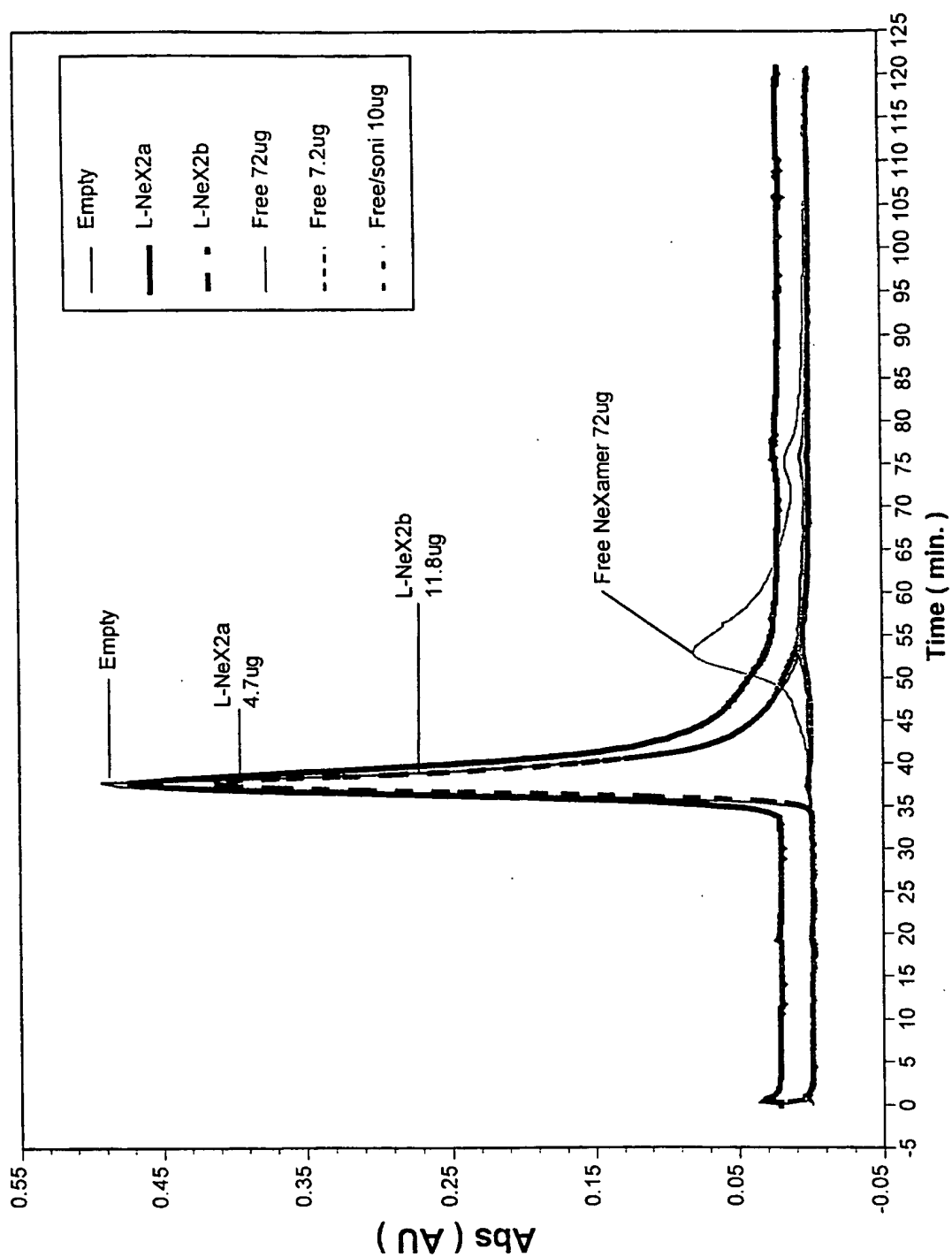


Figure 2

25/34

- pk 59 NX232 (0.892 mg/kg)
- pk 61 NX229 (0.892 mg/kg)
- ◇ pk 62 NX253-liposome (0.5 mg/kg)
- pk 63 NX256-PEG-20K (1 mg/kg)
- △ pk 64 NX253 (0.757 mg/kg)

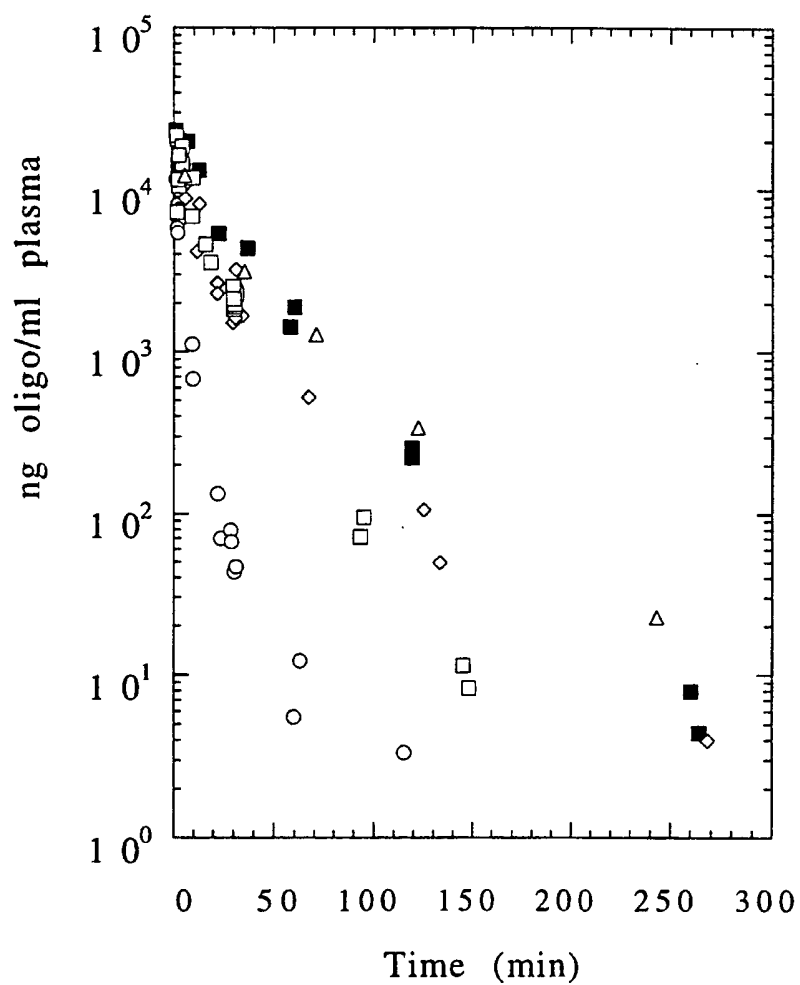


FIGURE 3

26/34

- ▲ pk 53 NX213 (0.55 mg/kg)
- pk 70 NX268 (1 mg/kg)
- △ pk 74 NX278 (1 mg/kg)
- pk 79 NX278 + liposome (1 mg/kg)
- ◇ pk 80 JW986 - PEG 2OK (1 mg/kg)
- ◆ pk 81 NX213 liposome encap. (1 mg/kg)
- pk 90 NX244 (1mg/kg)

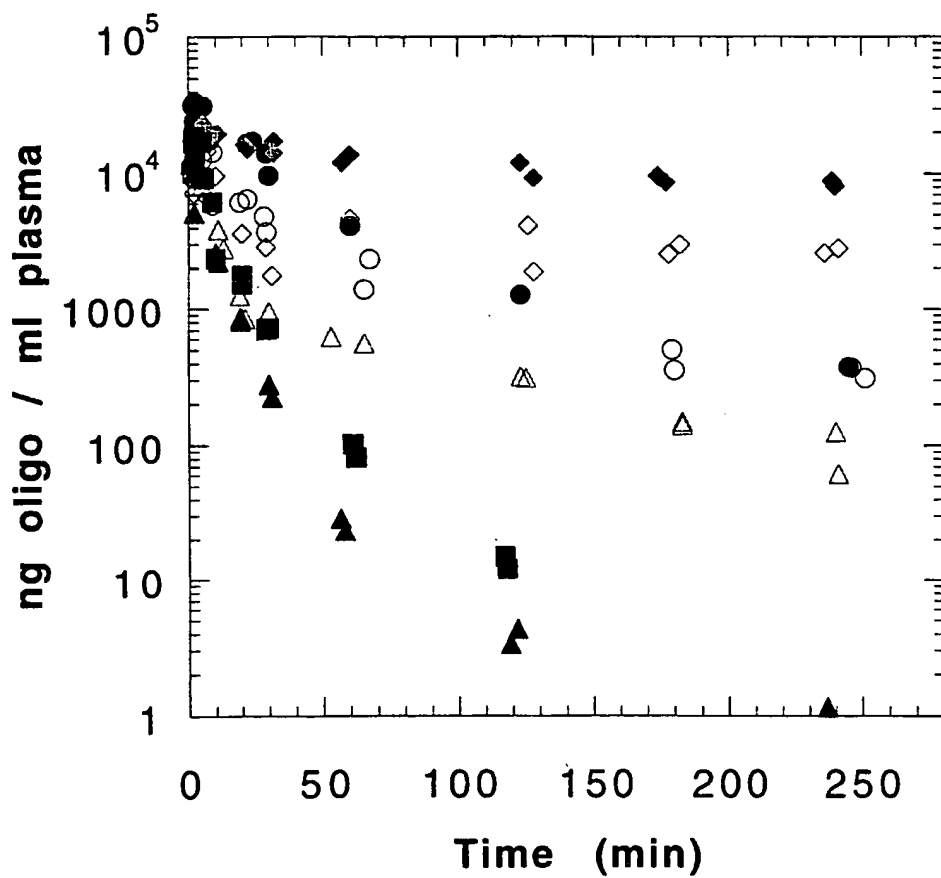


FIGURE 4

27/34

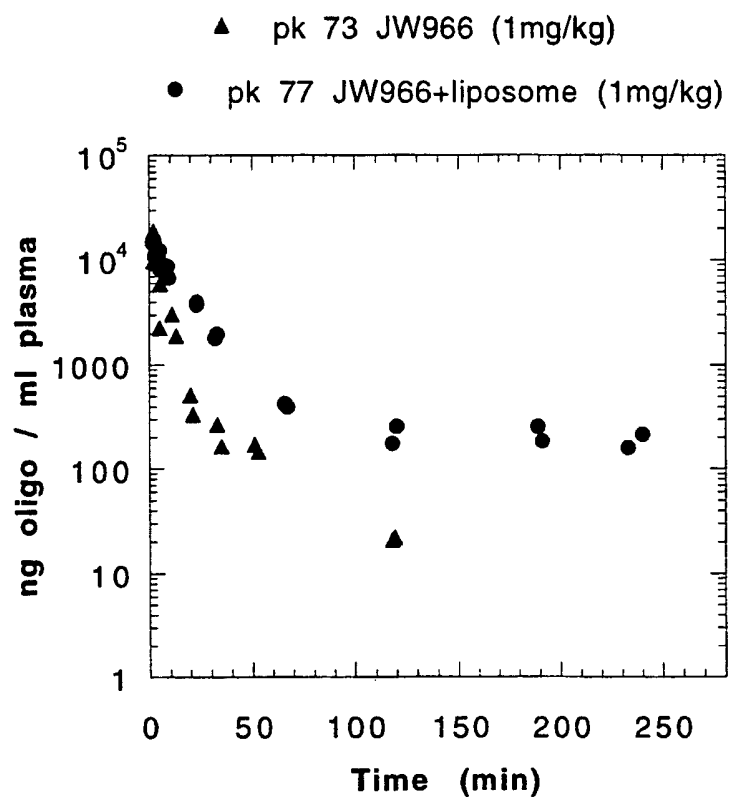


FIGURE 5

28/34

- ▲ pk 85 NX268 + PEG-liposome (1 mg/kg)
- pk 86 NX268 + liposome (1 mg/kg)

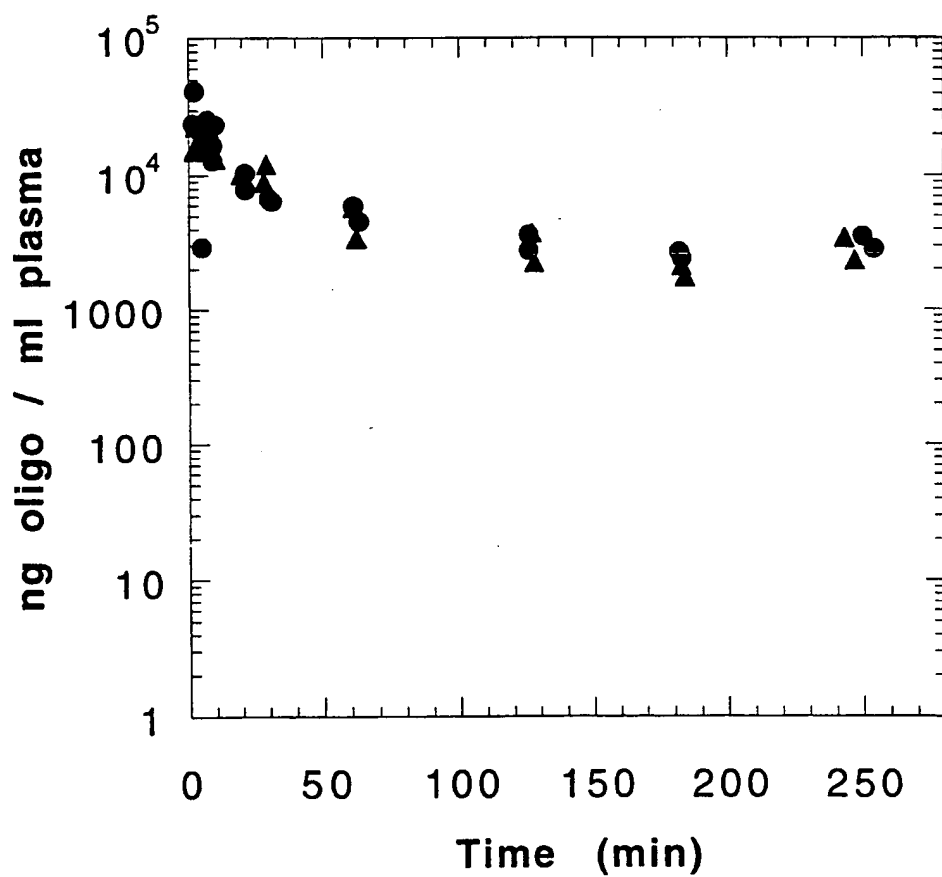


FIGURE 6

29/34

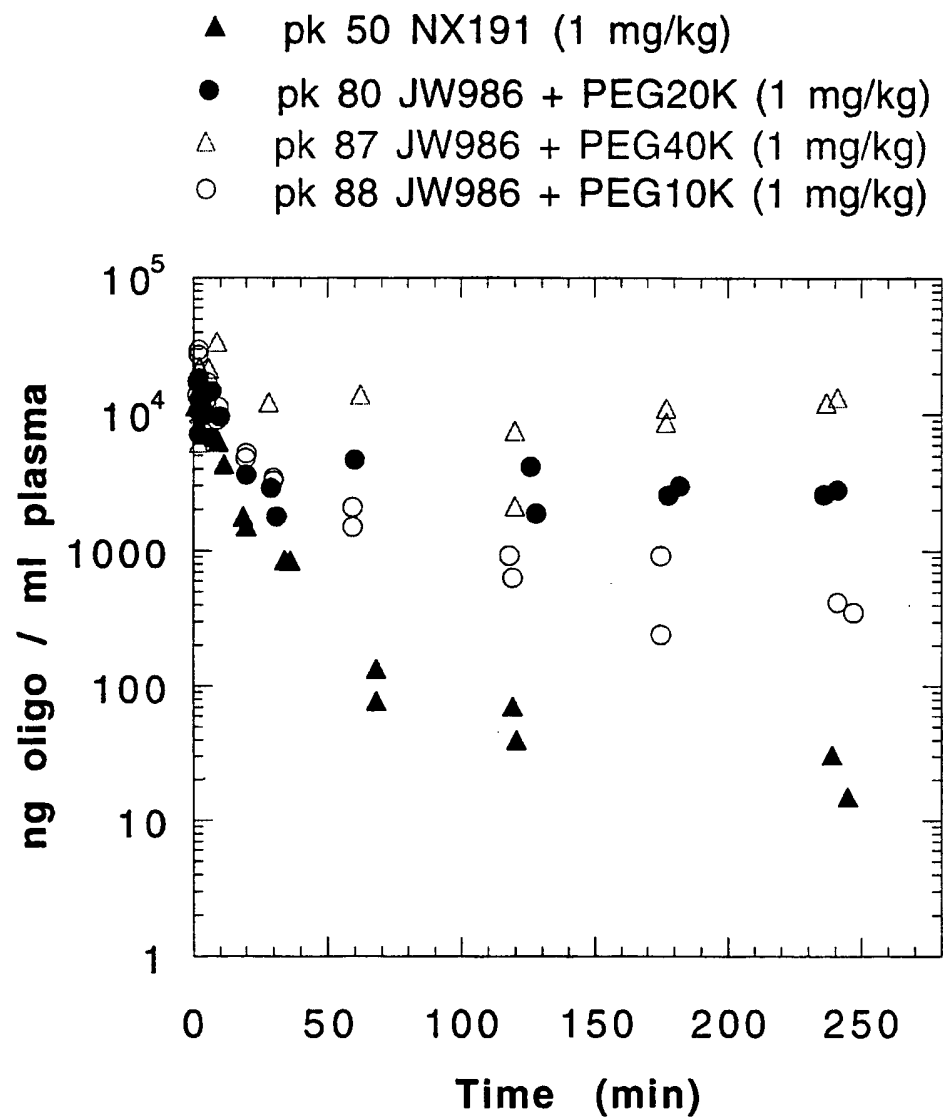


FIGURE 7



30/34

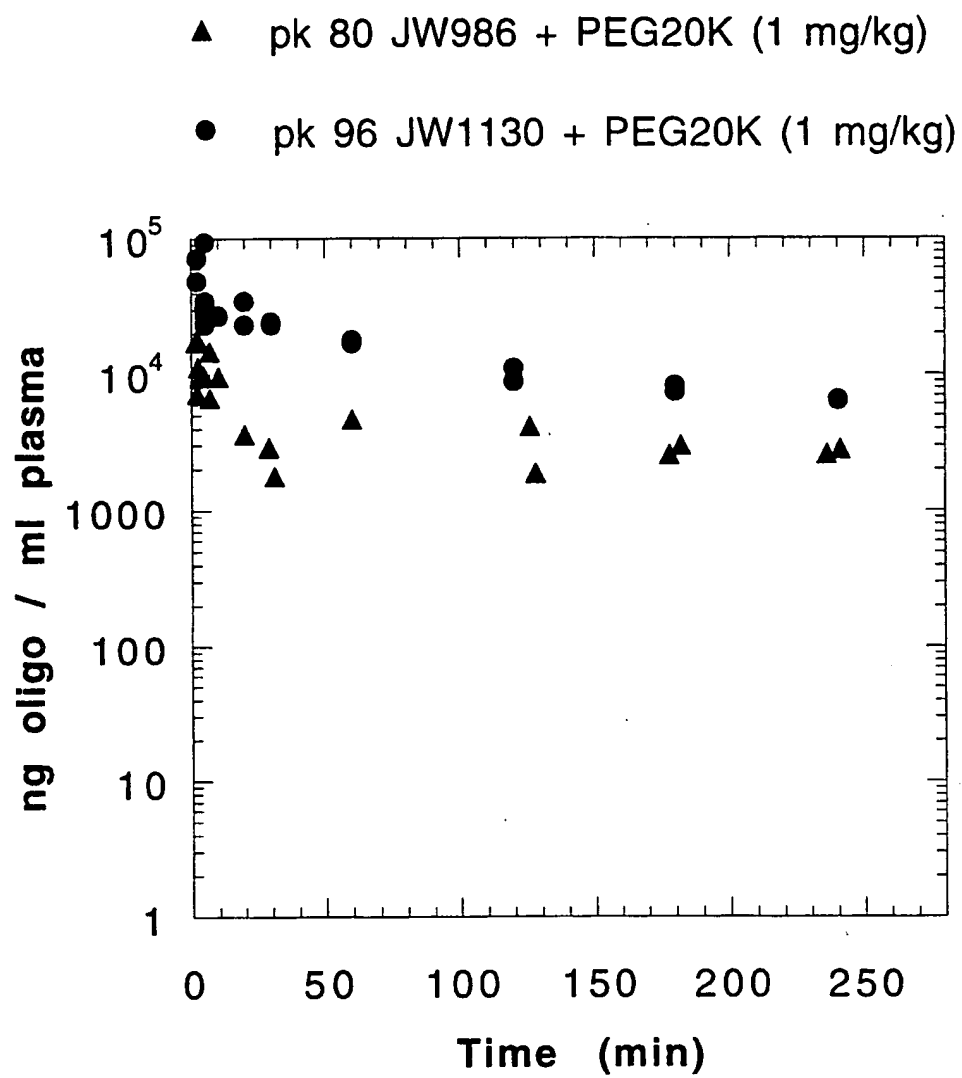


FIGURE 8

31/34

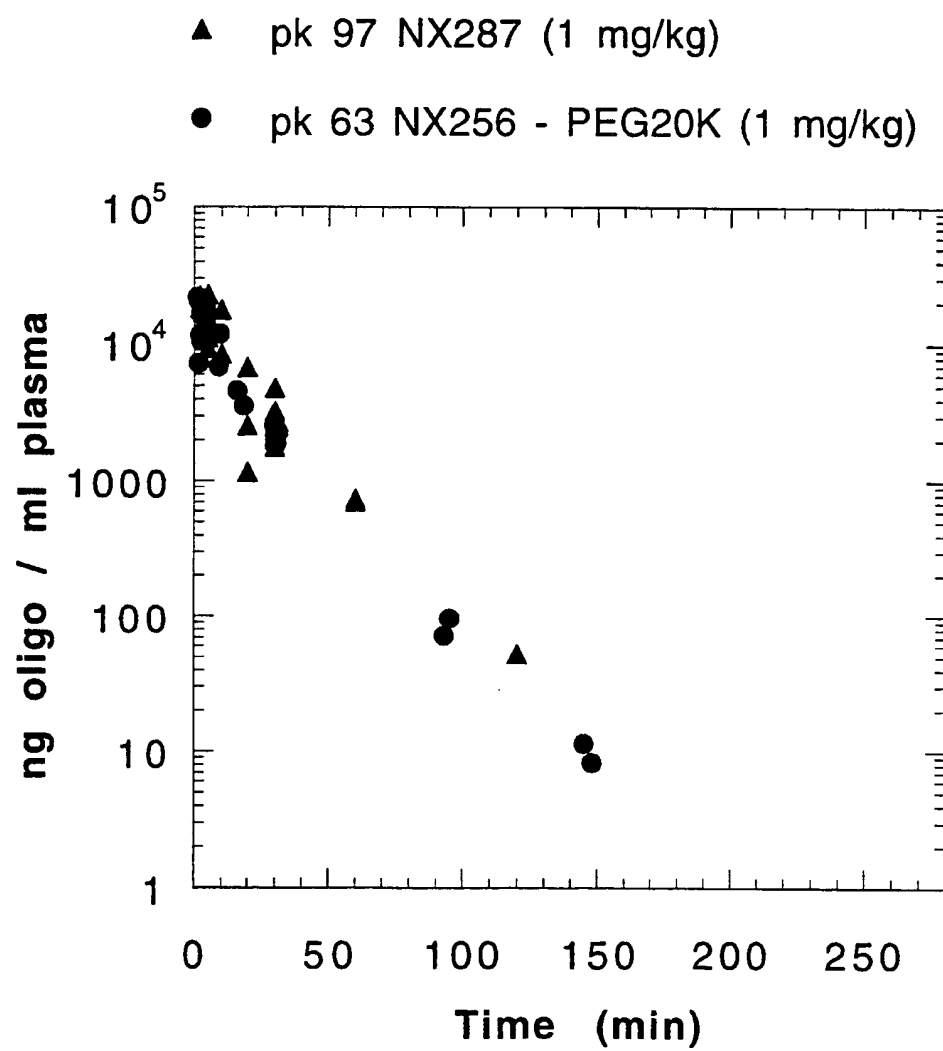


FIGURE 9

32/34

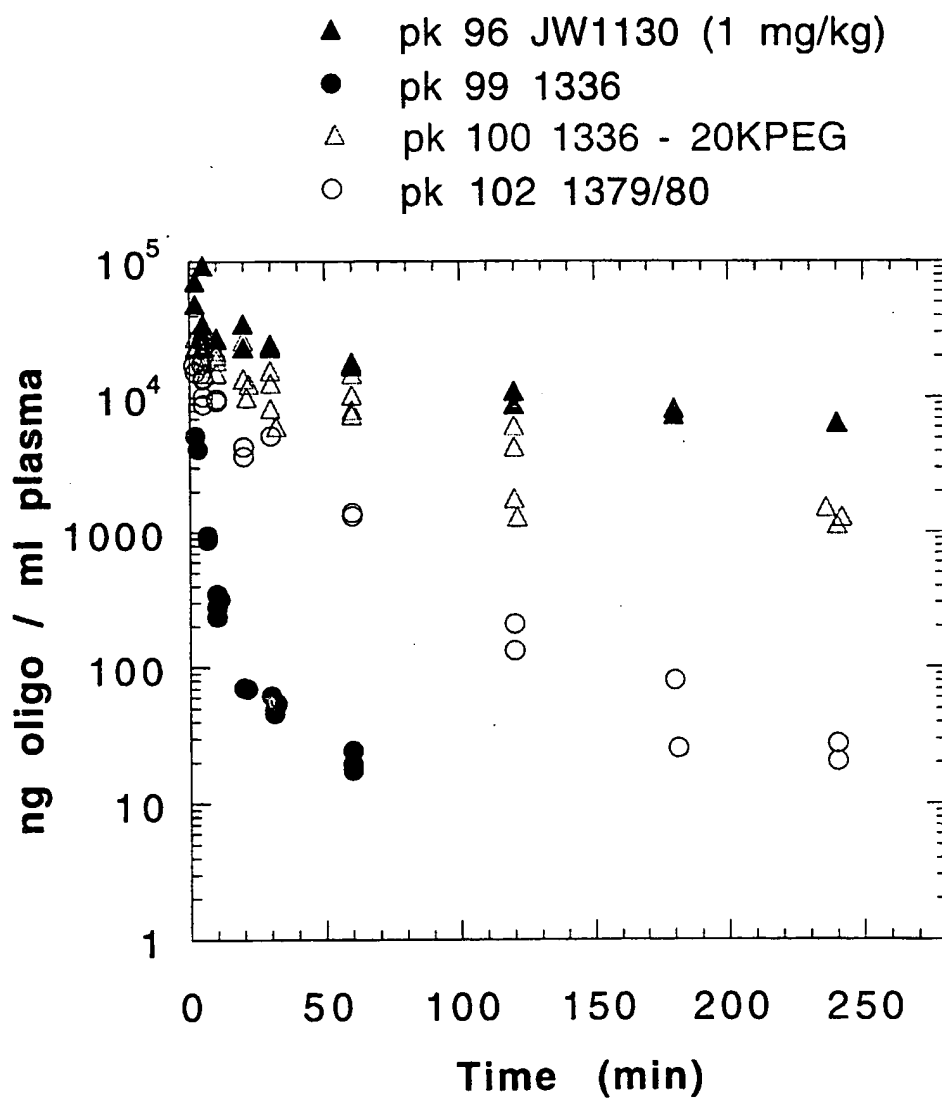


FIGURE 10

33/34

Nx232 + DSPC/CH/DSPG: 89/10/1, 87.5/10/2.5, or 85/10/5

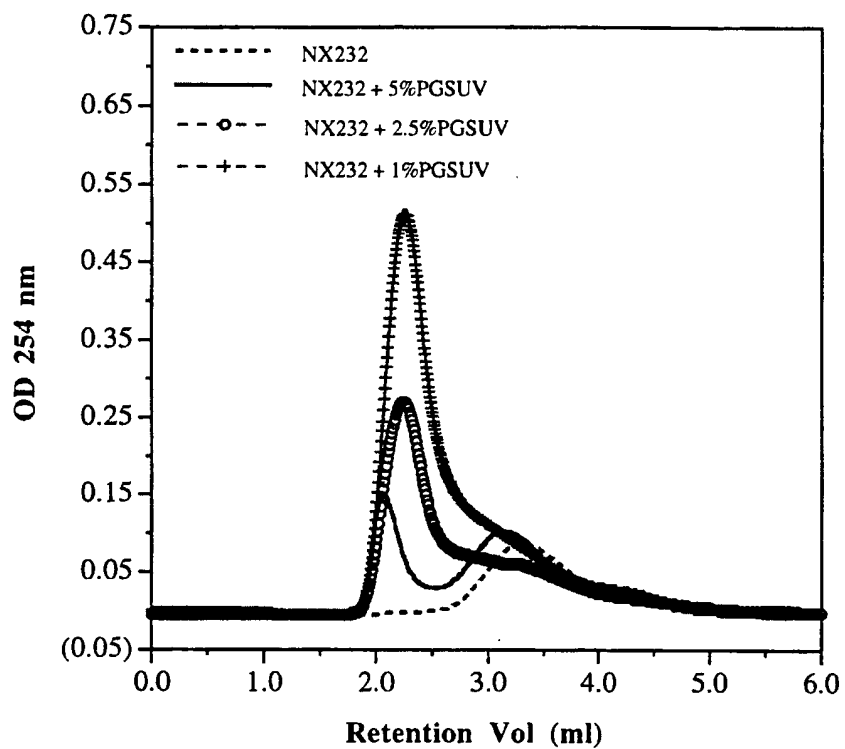


FIGURE 11

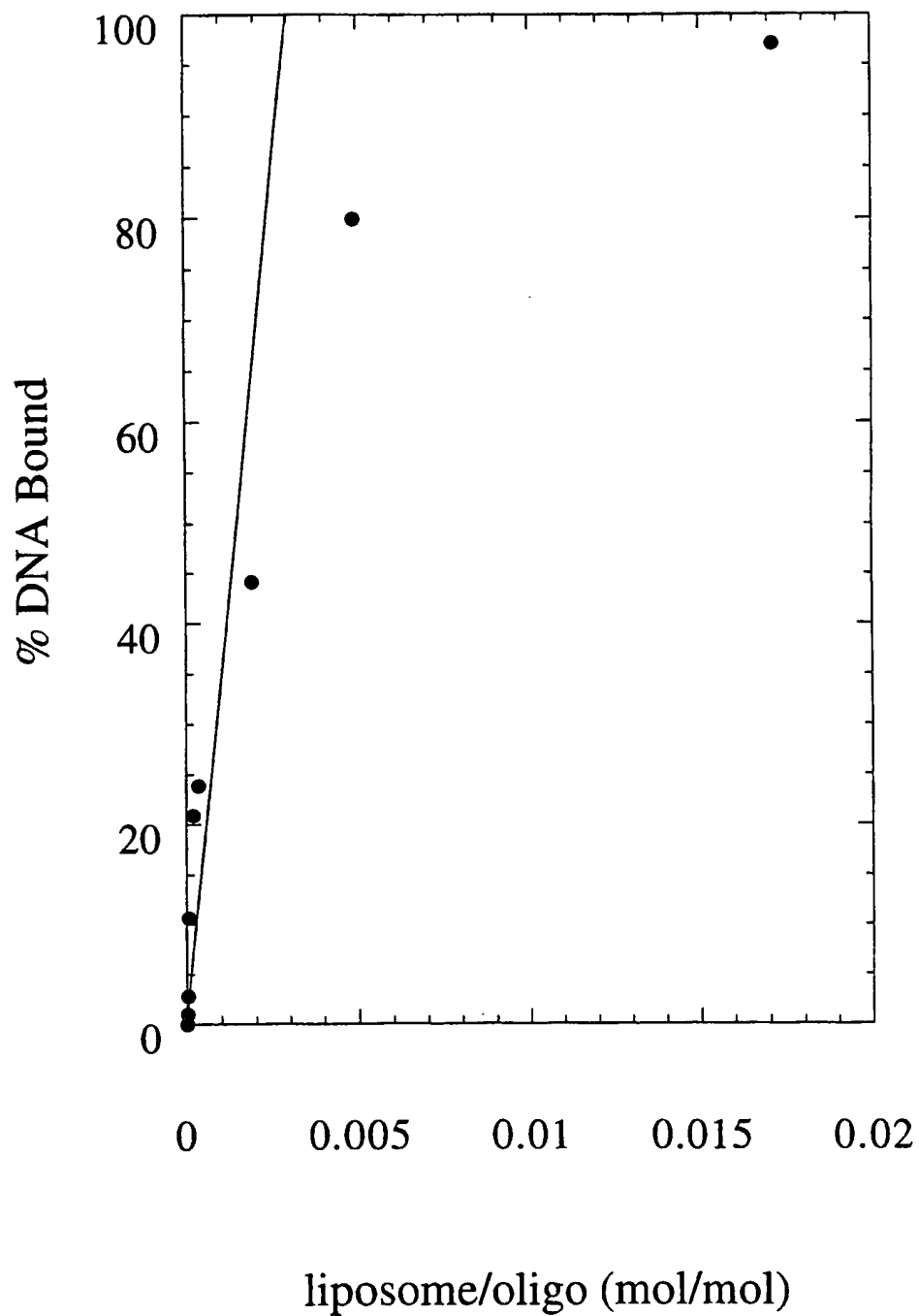


FIGURE 12

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/06171

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C07H 21/02, 21/04; C12P 19/34; C12Q 1/68

US CL : 435/6, 91.2; 536/22.1

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.2; 536/22.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,270,163 A (GOLD ET AL.) 14 DECEMBER 1993, COLUMNS 5, 11, LINES 15-33, 13-14	1-6,-20-22, 38-46, 66-69
Y	WO 92/14843 A1 (GILEAD SCIENCES, INC.) 03 SEPTEMBER 1992, PAGES 15-17, 29-34, 57-62	1-66, 20-22, 38-46, 66-69

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	* & * document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

25 JULY 1996

Date of mailing of the international search report

05 SEP 1996

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Authorized officer

STEPHANIE W. ZITOMER, Ph.D.

Facsimile No. (703) 305-3230

Telephone No. (703) 308-0196

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/06171

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  
1-6, 20-22, 38-46, and 66-69

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/06171

### BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains claims directed to more than one species and subspecies of the generic invention. These species are deemed to lack Unity of Invention because they are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for more than one species to be examined, the appropriate additional examination fees must be paid. The species are as follows:

Species A: Complex of Nucleic Acid Ligand and High Molecular Weight Compound, claims 1-6, 20-22, 33-46, 62-69

Subspecies 1: Polyethylene Glycol, claims 5, 6, 20-22, 38-40

Subspecies 2: Dextran, claim 5

Subspecies 3: Albumin, claim 5

Subspecies 4: Magnetite, claim 5

Species B: Complex of Nucleic Acid Ligand and Lipophilic Compound, claims 1-3, 7-19, 23-37, 47-61, 67-69

Subspecies 1: Cholesterol, claims 7-9, 11-13

Subspecies 2: Dialkyl Glycerol, claims 10, 17-19

Subspecies 3: Diacyl Glycerol, claim 8

Subspecies 4: Lipid Construct, claims 14-16, 23-32





## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>A61K 37/22, 31/70, 47/48, C07H 21/02, 21/04, C12P 19/34, C12Q 1/68</b>		<b>A1</b>	(11) International Publication Number: <b>WO 98/18480</b>
			(43) International Publication Date: 7 May 1998 (07.05.98)
(21) International Application Number: PCT/US97/18944 (22) International Filing Date: 17 October 1997 (17.10.97) (30) Priority Data: 08/739,109      25 October 1996 (25.10.96)      US 08/870,930      6 June 1997 (06.06.97)      US 08/897,351      21 July 1997 (21.07.97)      US (71) Applicant (for all designated States except US): NEXSTAR PHARMACEUTICALS, INC. [US/US]; 2860 Wilderness Place, Boulder, CO 80301 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): JANJIC, Nebojsa [US/US]; 6973 Carter Trail, Boulder, CO 80301 (US). GOLD, Larry [US/US]; 1033 5th Street, Boulder, CO 80302 (US). SCHMIDT, Paul, G. [US/US]; P.O. Box 1125, Niwot, CO 80544 (US). VARGESE, Chandra [IN/US]; 5295 East 117th Avenue, Thornton, CO 80233 (US). WILLIS, Michael [US/US]; 786 West Fir Court, Louisville, CO 80027 (US). (74) Agents: SWANSON, Barry, J. et al.; Swanson & Bratschun, L.L.C., Suite 200, 8400 East Prentice Avenue, Englewood, CO 80111 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>	
(54) Title: VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) NUCLEIC ACID LIGAND COMPLEXES			
(57) Abstract .  This invention discloses a method for preparing a complex comprised of a VEGF Nucleic Acid Ligand and a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound by identifying a VEGF Nucleic Acid Ligand by SELEX methodology and associating the VEGF Nucleic Acid Ligand with a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound. The invention further discloses Complexes comprising one or more VEGF Nucleic Acid Ligands in association with a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound. The invention further includes a Lipid construct comprising a VEGF Nucleic Acid Ligand or Complex and methods for making the same.			

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

5

**VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF)  
NUCLEIC ACID LIGAND COMPLEXES**

10

**FIELD OF THE INVENTION**

15

Described herein are high affinity 2' Fluoro (2'-F) pyrimidine RNA ligands to vascular endothelial growth factor (VEGF). The method utilized herein for identifying such Nucleic Acid Ligands is called SELEX, an acronym for Systematic Evolution of Ligands by Exponential enrichment. Further included in this invention is a method for preparing a therapeutic or diagnostic Complex comprised of a VEGF Nucleic Acid Ligand and a Non-Immunogenic, High Molecular Weight Compound or a Lipophilic Compound by identifying a VEGF Nucleic Acid Ligand by SELEX methodology and covalently linking the VEGF Nucleic Acid Ligand with a Non-Immunogenic, High Molecular Weight Compound or a Lipophilic Compound. The invention further includes Complexes comprised of one or more VEGF Nucleic Acid Ligand and a Non-Immunogenic, High Molecular Weight Compound or a Lipophilic Compound. The invention further relates to improving the Pharmacokinetic Properties of a VEGF Nucleic Acid Ligand by covalently linking the VEGF Nucleic Acid Ligand with a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound to form a Complex. The invention further relates to improving the Pharmacokinetic Properties of a VEGF Nucleic Acid Ligand by using a Lipid Construct comprising a VEGF Nucleic Acid Ligand or a Complex comprising a VEGF Nucleic Acid Ligand and a Non-Immunogenic, High Molecular Weight Compound

30

or Lipophilic Compound. This invention further relates to a method for targeting a therapeutic or diagnostic agent to a biological target that is expressing VEGF by associating the agent with a Complex comprised of a VEGF Nucleic Acid Ligand and a Lipophilic Compound or Non-Immunogenic, High Molecular Weight Compound, wherein the  
5 Complex is further associated with a Lipid Construct and the VEGF Nucleic Acid Ligand is further associated with the exterior of the Lipid Construct.

## **BACKGROUND OF THE INVENTION**

### **A. SELEX**

10 The dogma for many years was that nucleic acids had primarily an informational role. Through a method known as Systematic Evolution of Ligands by EXponential enrichment, termed SELEX, it has become clear that nucleic acids have three dimensional structural diversity not unlike proteins. SELEX is a method for the *in vitro* evolution of nucleic acid molecules with highly specific binding to target molecules and is described in  
15 United States Patent Application Serial No. 07/536,428, filed June 11, 1990, entitled "Systematic Evolution of Ligands by Exponential Enrichment," now abandoned, United States Patent Application Serial No. 07/714,131, filed June 10, 1991, entitled "Nucleic Acid Ligands," now United States Patent No. 5,475,096, United States Patent Application Serial No. 07/931,473, filed August 17, 1992, entitled "Nucleic Acid Ligands," now United States  
20 Patent No. 5,270,163 (see also WO 91/19813), each of which is specifically incorporated by reference herein. Each of these applications, collectively referred to herein as the SELEX Patent Applications, describes a fundamentally novel method for making a Nucleic Acid Ligand to any desired target molecule. The SELEX process provides a class of products which are referred to as Nucleic Acid Ligands, each ligand having a unique sequence, and  
25 which has the property of binding specifically to a desired target compound or molecule. Each SELEX-identified Nucleic Acid Ligand is a specific ligand of a given target compound or molecule. SELEX is based on the unique insight that Nucleic Acids have sufficient capacity for forming a variety of two- and three-dimensional structures and sufficient chemical versatility available within their monomers to act as ligands (form

specific binding pairs) with virtually any chemical compound, whether monomeric or polymeric. Molecules of any size or composition can serve as targets.

The SELEX method involves selection from a mixture of candidate oligonucleotides and step-wise iterations of binding, partitioning and amplification, using  
5 the same general selection scheme, to achieve virtually any desired criterion of binding affinity and selectivity. Starting from a mixture of Nucleic Acids, preferably comprising a segment of randomized sequence, the SELEX method includes steps of contacting the mixture with the target under conditions favorable for binding, partitioning unbound  
10 Nucleic Acids from those Nucleic Acids which have bound specifically to target molecules, dissociating the Nucleic Acid-target complexes, amplifying the Nucleic Acids dissociated from the Nucleic Acid-target complexes to yield a ligand-enriched mixture of Nucleic Acids, then reiterating the steps of binding, partitioning, dissociating and amplifying through as many cycles as desired to yield highly specific high affinity Nucleic Acid  
15 Ligands to the target molecule.

It has been recognized by the present inventors that the SELEX method  
15 demonstrates that Nucleic Acids as chemical compounds can form a wide array of shapes, sizes and configurations, and are capable of a far broader repertoire of binding and other functions than those displayed by Nucleic Acids in biological systems.

The present inventors have recognized that SELEX or SELEX-like processes could  
20 be used to identify Nucleic Acids which can facilitate any chosen reaction in a manner similar to that in which Nucleic Acid Ligands can be identified for any given target. In theory, within a Candidate Mixture of approximately  $10^{13}$  to  $10^{18}$  Nucleic Acids, the present inventors postulate that at least one Nucleic Acid exists with the appropriate shape to facilitate each of a broad variety of physical and chemical interactions.

25 The basic SELEX method has been modified to achieve a number of specific objectives. For example, United States Patent Application Serial No. 07/960,093, filed October 14, 1992, entitled "Method for Selecting Nucleic Acids on the Basis of Structure," describes the use of SELEX in conjunction with gel electrophoresis to select Nucleic Acid molecules with specific structural characteristics, such as bent DNA. United States Patent  
30 Application Serial No. 08/123,935, filed September 17, 1993, entitled "Photoselection of

Nucleic Acid Ligands," describes a SELEX based method for selecting Nucleic Acid Ligands containing photoreactive groups capable of binding and/or photocrosslinking to and/or photoinactivating a target molecule. United States Patent Application Serial No. 08/134,028, filed October 7, 1993, entitled "High-Affinity Nucleic Acid Ligands That  
5 Discriminate Between Theophylline and Caffeine," now United States Patent No. 5,580,737, describes a method for identifying highly specific Nucleic Acid Ligands able to discriminate between closely related molecules, which can be non-peptidic, termed Counter-SELEX. United States Patent Application Serial No. 08/143,564, filed October 25, 1993, entitled "Systematic Evolution of Ligands by EXponential Enrichment: Solution  
10 SELEX," now United States Patent No. 5,567,588, describes a SELEX-based method which achieves highly efficient partitioning between oligonucleotides having high and low affinity for a target molecule.

The SELEX method encompasses the identification of high-affinity Nucleic Acid Ligands containing modified nucleotides conferring improved characteristics on the ligand,  
15 such as improved *in vivo* stability or improved delivery characteristics. Examples of such modifications include chemical substitutions at the ribose and/or phosphate and/or base positions. SELEX-identified Nucleic Acid Ligands containing modified nucleotides are described in United States Patent Application Serial No. 08/117,991, filed September 8, 1993, entitled "High Affinity Nucleic Acid Ligands Containing Modified Nucleotides,"  
20 now United States Patent No. 5,660,985, that describes oligonucleotides containing nucleotide derivatives chemically modified at the 5- and 2'-positions of pyrimidines. United States Patent Application Serial No. 08/134,028, *supra*, describes highly specific Nucleic Acid Ligands containing one or more nucleotides modified with 2'-amino (2'-NH<sub>2</sub>), 2'-fluoro (2'-F), and/or 2'-O-methyl (2'-OMe). United States Patent Application Serial No.  
25 08/264,029, filed June 22, 1994, entitled "Novel Method of Preparation of 2' Modified Pyrimidine Intramolecular Nucleophilic Displacement," describes oligonucleotides containing various 2'-modified pyrimidines.

The SELEX method encompasses combining selected oligonucleotides with other selected oligonucleotides and non-oligonucleotide functional units as described in United  
30 States Patent Application Serial No. 08/284,063, filed August 2, 1994, entitled "Systematic

Evolution of Ligands by Exponential Enrichment: Chimeric SELEX," now United States Patent No. 5,637,459, and United States Patent Application Serial No. 08/234,997, filed April 28, 1994, entitled "Systematic Evolution of Ligands by Exponential Enrichment: Blended SELEX," respectively. These applications allow the combination of the broad  
5 array of shapes and other properties, and the efficient amplification and replication properties, of oligonucleotides with the desirable properties of other molecules.

The SELEX method further encompasses combining selected Nucleic Acid Ligands with Lipophilic Compounds or Non-Immunogenic, High Molecular Weight Compounds in a diagnostic or therapeutic Complex as described in United States Patent Application Serial  
10 No. 08/434,465, filed May 4, 1995, entitled "Nucleic Acid Complexes." VEGF Nucleic Acid Ligands that are associated with a Lipophilic Compound, such as diacyl glycerol or dialkyl glycerol, in a diagnostic or therapeutic complex are described in United States Patent Application Serial No. 08/739,109, filed October 25, 1996, entitled "Vascular Endothelial Growth Factor (VEGF) Nucleic Acid Ligand Complexes." VEGF Nucleic  
15 Acid Ligands that are associated with a High Molecular Weight, Non-Immunogenic Compound, such as Polyethyleneglycol, or a Lipophilic Compound, such as Glycerolipid, phospholipid, or glycerol amide lipid, in a diagnostic or therapeutic complex are described in United States Patent Application Serial No. 08/897,351, filed July 21, 1997, entitled "Vascular Endothelial Growth Factor (VEGF) Nucleic Acid Complexes." Each of the  
20 above described patent applications which describe modifications of the basic SELEX procedure are specifically incorporated by reference herein in their entirety.

## B. LIPID CONSTRUCTS

Lipid Bilayer Vesicles are closed, fluid-filled microscopic spheres which are  
25 formed principally from individual molecules having polar (hydrophilic) and non-polar (lipophilic) portions. The hydrophilic portions may comprise phosphato, glycerylphosphato, carboxy, sulfato, amino, hydroxy, choline or other polar groups. Examples of lipophilic groups are saturated or unsaturated hydrocarbons such as alkyl, alkenyl or other lipid groups. Sterols (e.g., cholesterol) and other pharmaceutically  
30 acceptable adjuvants (including anti-oxidants like alpha-tocopherol) may also be included

to improve vesicle stability or confer other desirable characteristics.

Liposomes are a subset of these bilayer vesicles and are comprised principally of phospholipid molecules that contain two hydrophobic tails consisting of fatty acid chains. Upon exposure to water, these molecules spontaneously align to form spherical, bilayer  
5 membranes with the lipophilic ends of the molecules in each layer associated in the center of the membrane and the opposing polar ends forming the respective inner and outer surface of the bilayer membrane(s). Thus, each side of the membrane presents a hydrophilic surface while the interior of the membrane comprises a lipophilic medium. These membranes may be arranged in a series of concentric, spherical membranes  
10 separated by thin strata of water, in a manner not dissimilar to the layers of an onion, around an internal aqueous space. These multilamellar vesicles (MLV) can be converted into small or Unilamellar Vesicles (UV), with the application of a shearing force.

The therapeutic use of liposomes includes the delivery of drugs which are normally toxic in the free form. In the liposomal form, the toxic drug is occluded, and  
15 may be directed away from the tissues sensitive to the drug and targeted to selected areas. Liposomes can also be used therapeutically to release drugs over a prolonged period of time, reducing the frequency of administration. In addition, liposomes can provide a method for forming aqueous dispersions of hydrophobic or amphiphilic drugs, which are normally unsuitable for intravenous delivery.

20 In order for many drugs and imaging agents to have therapeutic or diagnostic potential, it is necessary for them to be delivered to the proper location in the body, and the liposome can thus be readily injected and form the basis for sustained release and drug delivery to specific cell types, or parts of the body. Several techniques can be employed to use liposomes to target encapsulated drugs to selected host tissues, and away  
25 from sensitive tissues. These techniques include manipulating the size of the liposomes, their net surface charge, and their route of administration. MLVs, primarily because they are relatively large, are usually rapidly taken up by the reticuloendothelial system (principally the liver and spleen). UVs, on the other hand, have been found to exhibit increased circulation times, decreased clearance rates and greater biodistribution relative  
30 to MLVs.



Passive delivery of liposomes involves the use of various routes of administration, e.g., intravenous, subcutaneous, intramuscular and topical. Each route produces differences in localization of the liposomes. Two common methods used to direct liposomes actively to selected target areas involve attachment of either antibodies or specific receptor ligands to the surface of the liposomes. Antibodies are known to have a high specificity for their corresponding antigen and have been attached to the surface of liposomes, but the results have been less than successful in many instances. Some efforts, however, have been successful in targeting liposomes to tumors without the use of antibodies, see, for example, U.S. Patent No. 5,019,369, U.S. Patent No. 5,441,745, or U.S. Patent No. 5,435,989.

An area of development aggressively pursued by researchers is the delivery of agents not only to a specific cell type but into the cell's cytoplasm and, further yet, into the nucleus. This is particularly important for the delivery of biological agents such as DNA, RNA, ribozymes and proteins. A promising therapeutic pursuit in this area involves the use of antisense DNA and RNA oligonucleotides for the treatment of disease. However, one major problem encountered in the effective application of antisense technology is that oligonucleotides in their phosphodiester form are quickly degraded in body fluids and by intracellular and extracellular enzymes, such as endonucleases and exonucleases, before the target cell is reached. Intravenous administration also results in rapid clearance from the bloodstream by the kidney, and uptake is insufficient to produce an effective intracellular drug concentration. Liposome encapsulation protects the oligonucleotides from the degradative enzymes, increases the circulation half-life and increases uptake efficiency as a result of phagocytosis of the Liposomes. In this way, oligonucleotides are able to reach their desired target and to be delivered to cells *in vivo*.

A few instances have been reported where researchers have attached antisense oligonucleotides to Lipophilic Compounds or Non-Immunogenic, High Molecular Weight Compounds. Antisense oligonucleotides, however, are only effective as intracellular agents. Antisense oligodeoxyribonucleotides targeted to the epidermal growth factor (EGF) receptor have been encapsulated into Liposomes linked to folate via

a polyethylene glycol spacer (folate-PEG-Liposomes) and delivered into cultured KB cells via folate receptor-mediated endocytosis (Wang *et al.* (1995) 92:3318-3322). In addition, alkylene diols have been attached to oligonucleotides (Weiss *et al.*, U.S. Patent No. 5,245,022). Furthermore, a Lipophilic Compound covalently attached to an antisense oligonucleotide has been demonstrated in the literature (EP 462 145 B1).

Loading of biological agents into liposomes can be accomplished by inclusion in the lipid formulation or loading into preformed liposomes. Passive anchoring of oligopeptide and oligosaccharide ligands to the external surface of liposomes has been described (Zalipsky *et al.* (1997) *Bioconjug. Chem.* 8:111:118).

10

### C. VEGF

The growth of new blood vessels from existing endothelium (angiogenesis) is tightly controlled in healthy adults by opposing effects of positive and negative regulators. Under certain pathological conditions, including proliferative retinopathies, rheumatoid arthritis, psoriasis and cancer, positive regulators prevail and angiogenesis contributes to disease progression (reviewed in Folkman (1995) *Nature Medicine* 1:27-31). In cancer, the notion that angiogenesis represents the rate limiting step of tumor growth and metastasis (Folkman (1971) *New Engl. J. Med.* 285:1182-1186) is now supported by considerable experimental evidence (reviewed in Aznavoorian *et al.* (1993) *Cancer* 71:1368-1383; Fidler and Ellis (1994) *Cell* 79:185-188; Folkman (1990) *J. Natl. Cancer Inst.* 82:4-6).

The quantity of blood vessels in tumor tissue is a strong negative prognostic indicator in breast cancer (Weidner *et al.* (1992) *J. Natl. Cancer Inst.* 84:1875-1887) prostate cancer (Weidner *et al.* (1993) *Am. J. Pathol.* 143:401-409), brain tumors (Li *et al.* (1994) *Lancet* 344:82-86), and melanoma (Foss *et al.* (1996) *Cancer Res.* 56:2900-2903).

A number of angiogenic growth factors have been described to date among which vascular endothelial growth factor (VEGF) appears to play a key role as a positive regulator of physiological and pathological angiogenesis (reviewed in (Brown *et al.* (1996) *Control of Angiogenesis* (Goldberg and Rosen, eds.) Birkhauser, Basel, *in press*; Thomas (1996) *J. Biol. Chem.* 271:603-606). VEGF is a secreted disulfide-linked homodimer that selectively stimulates endothelial cells to proliferate, migrate, and produce matrix-degrading enzymes

30

(Conn et al.(1990) Proc. Natl. Acad. Sci. USA 87:1323-1327); Ferrara and Henzel (1989) Biochem. Biophys. Res. Commun. 161:851-858); Gospodarowicz et al.(1989) Proc. Natl. Acad. Sci. USA 73:11-7315); Pepper et al.(1991) Biochem. Biophys. Res. Commun. 181:902-906; Unemori et al.(1992) J. Cell. Physiol. 153:557-562), all of which are

5 processes required for the formation of new vessels. In addition to being the only known endothelial cell specific mitogen, VEGF is unique among angiogenic growth factors in its ability to induce a transient increase in blood vessel permeability to macromolecules (hence its original and alternative name, vascular permeability factor, VPF) (Dvorak et al.(1979) J. Immunol. 122:166-174; Senger et al.(1983) Science 219:983-985; Senger et al.(1986)

10 Cancer Res. 46:5629-5632). Increased vascular permeability and the resulting deposition of plasma proteins in the extravascular space assists the new vessel formation by providing a provisional matrix for the migration of endothelial cells (Dvorak et al.(1995) Am. J. Pathol. 146:1029-1039). Hyperpermeability is indeed a characteristic feature of new vessels, including those associated with tumors (Dvorak et al.(1995) Am. J. Pathol. 146:1029-1039).

15 Furthermore, compensatory angiogenesis induced by tissue hypoxia is now known to be mediated by VEGF (Levy et al.(1996) J. Biol. Chem. 274:2746-2753); Shweiki et al. (1992) Nature 359:843-845).

VEGF occurs in four forms (VEGF-121, VEGF-165, VEGF-189, VEGF-206) as a result of alternative splicing of the VEGF gene (Houck et al. (1991) Mol. Endocrin. 5:1806-

20 1814; Tischer et al. (1991) J. Biol. Chem. 266:11947-11954). The two smaller forms are diffusable while the larger two forms remain predominantly localized to the cell membrane as a consequence of their high affinity for heparin. VEGF-165 also binds to heparin and is the most abundant form. VEGF-121, the only form that does not bind to heparin, appears to have a lower affinity for the receptors (Gitay-Goren et al. (1996) J. Biol. Chem. 271:5519-

25 5523) as well as lower mitogenic potency (Keyt et al. (1996) J. Biol. Chem. 271:7788-7795). The biological effects of VEGF are mediated by two tyrosine kinase receptors (Flt-1 and Flk-1/KDR) whose expression is highly restricted to cells of endothelial origin (de Vries et al. (1992) Science 255:989-991; Millauer et al. (1993) Cell 72:835-846; Terman et al. (1991) Oncogene 6:519-524). While the expression of both functional receptors is

30 required for high affinity binding, the chemotactic and mitogenic signaling in endothelial

cells appears to occur primarily through the KDR receptor (Park et al. (1994) J. Biol. Chem. 269:25646-25654; Seetharam et al. (1995) Oncogene 10:135-147; Waltenberger et al. (1994) J. Biol. Chem. 269:26988-26995). The importance of VEGF and VEGF receptors for the development of blood vessels has recently been demonstrated in mice lacking a single  
5 allele for the VEGF gene (Carmeliet et al. (1996) Nature 380:435-439; Ferrara et al. (1996) Nature 380:439-442) or both alleles of the Flt-1 (Fong et al. (1995) 376:66-70) or Flk-1 genes (Shalaby et al. (1995) Nature 376:62-66). In each case, distinct abnormalities in vessel formation were observed resulting in embryonic lethality.

VEGF is produced and secreted in varying amounts by virtually all tumor cells  
10 (Brown et al. (1997) Regulation of Angiogenesis (Goldberg and Rosen, Eds.) Birkhauser, Basel, pp. 233-269). Direct evidence that VEGF and its receptors contribute to tumor growth was recently obtained by a demonstration that the growth of human tumor xenografts in nude mice could be inhibited by neutralizing antibodies to VEGF (Kim et al. (1993) Nature 362:841-844), by the expression of dominant-negative VEGF receptor flk-1  
15 (Millauer et al. (1996) Cancer Res. 56:1615-1620; Millauer et al. (1994) Nature 367:576-579), by low molecular weight inhibitors of Flk-1 tyrosine kinase activity (Strawn et al. (1966) Cancer Res. 56:3540-3545), or by the expression of antisense sequence to VEGF mRNA (Saleh et al. (1996) Cancer Res. 56:393-401). Importantly, the incidence of tumor metastases was also found to be dramatically reduced by VEGF antagonists (Claffey et al.  
20 (1996) Cancer Res. 56:172-181).

In addition to their use as anticancer agents, VEGF inhibitors may be useful in a wide variety of proliferative diseases characterized by excessive angiogenesis, including psoriasis, ocular disorders, collagen vascular diseases and rheumatoid arthritis. Although most tumor types are known to produce VEGF, until recently none has been shown to  
25 express functional VEGF receptors. It has been shown that Kaposi's Sarcoma (KS) cells not only produce abundant amounts of VEGF but also express functional VEGF receptors and therefore use VEGF for autocrine growth. Kaposi's sarcoma is typically treated with conventional antimetabolic drugs. However, a major shortcoming of the use of chemotherapy in KS patients is the accompanying induction of immunosuppression which  
30 has serious consequences in patients whose immune system is already compromised. The

need for alternative therapies is especially great in early stages of the disease where KS lesions begin to appear but the patients otherwise feel fairly healthy. In this regard, encapsulation of chemotherapeutic drugs such as daunorubicin into liposomes has recently proved to be a promising method of minimizing side effects of chemotherapy while  
5 maintaining anti-tumor efficacy. Drugs with low toxicity that selectively target activated cells of endothelial origin, such as the Nucleic Acid Ligand VEGF antagonists described here, would be an enormous asset in the treatment of KS.

Other areas of potential clinical utility for the VEGF Nucleic Acid Ligands are ocular disorders characterized by excessive angiogenesis. Examples of such diseases are  
10 macular degeneration and diabetic retinopathy. In macular degeneration, progressive choroidal angiogenesis beneath the macula (a part of the retina responsible for the highest visual acuity) interferes with vision. In diabetic retinopathy, angiogenesis in the retina interferes with vision. While the initial stimuli that initiate blood vessel growth in macular degeneration and diabetic retinopathy are not known at present, VEGF appears to be a  
15 key angiogenesis inducer (Lopez, P. F. et al. (1996) Invest. Ophthalmol. Visual Science 37, 855-868; Kliffen, M. et al. (1997) Br. J. Ophthalmol. 81, 154-162; Kvant, A. et al. (1996) Invest. Ophthalmol. Visual Science 37, 1929-1934; Paques et al. (1997) Diabetes & Metabolism 23:125-130). Inhibitors of VEGF therefore may be useful in attenuating angiogenesis in macular degeneration.

20

### **SUMMARY OF THE INVENTION**

Described herein are high affinity 2' Fluoro (2'-F)-modified pyrimidine RNA ligands to vascular endothelial growth factor (VEGF). The method utilized herein for identifying such nucleic acid ligands is called SELEX, an acronym for Systematic  
25 Evolution of Ligands by Exponential enrichment. The ligands described herein were selected from an initial pool of about  $10^{14}$  RNA molecules randomized at 30 or 40 contiguous positions. Included herein are the evolved ligands that are shown in **Tables 2-6**. Further included in this invention is a method for preparing a Complex comprised of a VEGF Nucleic Acid Ligand and a Non-Immunogenic, High Molecular Weight Compound  
30 or Lipophilic Compound by the method comprising identifying a Nucleic Acid Ligand from

a Candidate Mixture of Nucleic Acids where the Nucleic Acid is a ligand of VEGF by the method of (a) contacting the Candidate Mixture of Nucleic Acids with VEGF, (b) partitioning between members of said Candidate Mixture on the basis of affinity to VEGF, and c) amplifying the selected molecules to yield a mixture of Nucleic Acids enriched for  
5 Nucleic Acid sequences with a relatively higher affinity for binding to VEGF, and covalently linking said identified VEGF Nucleic Acid Ligand with a Non-Immunogenic, High Molecular Weight Compound or a Lipophilic Compound. The invention further comprises a Complex comprised of a VEGF Nucleic Acid Ligand and a Non-Immunogenic, High Molecular Weight Compound or a Lipophilic Compound.

10 The invention further includes a Lipid Construct comprising a VEGF Nucleic Acid Ligand or a Complex. The present invention further relates to a method for preparing a Lipid Construct comprising a Complex wherein the Complex is comprised of a VEGF Nucleic Acid Ligand and a Lipophilic Compound.

In another embodiment, this invention provides a method for improving the  
15 pharmacokinetic properties of a VEGF Nucleic Acid Ligand by covalently linking the VEGF Nucleic Acid Ligand with a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound to form a Complex and administering the Complex to a patient. The invention further relates to a method for improving the pharmacokinetic properties of a VEGF Nucleic Acid Ligand by further associating the Complex with a Lipid Construct.

20 It is an object of the present invention to provide Complexes comprising one or more VEGF Nucleic Acid Ligands in association with one or more Non-Immunogenic, High Molecular Weight Compounds or Lipophilic Compounds and methods for producing the same. It is a further object of the present invention to provide Lipid Constructs comprising a Complex. It is a further object of the invention to provide one or more VEGF  
25 Nucleic Acid Ligands in association with one or more Non-Immunogenic, High Molecular Weight Compounds or Lipophilic Compounds with improved Pharmacokinetic Properties.

In embodiments of the invention directed to Complexes comprised of a VEGF Nucleic Acid Ligand and a Non-Immunogenic, High Molecular Weight Compound, it is preferred that the Non-Immunogenic, High Molecular Weight Compound is Polyalkylene  
30 Glycol, more preferably, polyethylene glycol (PEG). More preferably, the PEG has a

molecular weight of about 10-80K. Most preferably, the PEG has a molecular weight of about 20-45K. In embodiments of the invention directed to Complexes comprised of a VEGF Nucleic Acid Ligand and a Lipophilic Compound, it is preferred that the Lipophilic Compound is a glycerolipid. In the preferred embodiments of the invention, the Lipid Construct is preferably a Lipid Bilayer Vesicle and most preferably a Liposome. In the preferred embodiment, the VEGF Nucleic Acid Ligand is identified according to the SELEX method.

In embodiments of the invention directed to Complexes comprising a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound covalently linked to a VEGF Nucleic Acid Ligand or Ligands, the VEGF Nucleic Acid Ligand or Ligands can serve in a targeting capacity.

Additionally, the VEGF Nucleic Acid Ligand can be associated through Covalent or Non-Covalent Interactions with a Lipid Construct without being part of a Complex.

Furthermore, in embodiments of the invention directed to Lipid Constructs comprising a VEGF Nucleic Acid Ligand or a Non-Immunogenic, High Molecular Weight or Lipophilic Compound/ VEGF Nucleic Acid Ligand Complex where the Lipid Construct is of a type that has a membrane defining an interior compartment such as a Lipid Bilayer Vesicle, the VEGF Nucleic Acid Ligand or Complex in association with the Lipid Construct may be associated with the membrane of the Lipid Construct or encapsulated within the compartment. In embodiments where the VEGF Nucleic Acid Ligand is in association with the membrane, the VEGF Nucleic Acid Ligand can associate with the interior-facing or exterior-facing part of the membrane, such that the VEGF Nucleic Acid Ligand is projecting into or out of the vesicle. In certain embodiments, a VEGF Nucleic Acid Ligand Complex can be passively loaded onto the outside of a preformed Lipid Construct. In embodiments where the Nucleic Acid Ligand is projecting out of the Lipid Construct, the VEGF Nucleic Acid Ligand can serve in a targeting capacity.

In embodiments where the VEGF Nucleic Acid Ligand of the Lipid Construct serves in a targeting capacity, the Lipid Construct can have associated with it additional therapeutic or diagnostic agents. In one embodiment, the therapeutic or diagnostic agent is associated with the exterior of the Lipid Construct. In other embodiments, the therapeutic

or diagnostic agent is encapsulated in the Lipid Construct or associated with the interior of the Lipid Construct. In yet a further embodiment, the therapeutic or diagnostic agent is associated with the Complex. In one embodiment, the therapeutic agent is a drug. In an alternative embodiment, the therapeutic or diagnostic agent is one or more additional

5 Nucleic Acid Ligands.

It is a further object of the present invention to provide a method for inhibiting angiogenesis by the administration of a VEGF Nucleic Acid Ligand or a Complex comprising a VEGF Nucleic Acid Ligand and Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound or a Lipid Construct comprising the Complex of the present invention. It is yet a further object of the present invention to provide a method for inhibiting the growth of tumors by the administration of a VEGF Nucleic Acid Ligand or Complex comprising a VEGF Nucleic Acid Ligand and Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound or a Lipid Construct comprising a Complex of the present invention. It is yet a further object of the invention to provide a method for inhibiting Kaposi's Sarcoma by the administration of a VEGF Nucleic Acid Ligand or Complex comprising a VEGF Nucleic Acid Ligand and Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound or a Lipid Construct comprising a Complex of the present invention. It is yet a further object of the invention to provide a method for inhibiting macular degeneration by the administration of a VEGF Nucleic Acid Ligand or Complex comprising a VEGF Nucleic Acid Ligand and Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound or a Lipid Construct comprising a Complex of the present invention. It is yet a further object of the invention to provide a method for inhibiting diabetic retinopathy by the administration of a VEGF Nucleic Acid Ligand or Complex comprising a VEGF Nucleic Acid Ligand and Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound or a Lipid Construct comprising a Complex of the present invention.

It is a further object of the invention to provide a method for targeting a therapeutic or diagnostic agent to a biological target that is expressing VEGF by associating the agent with a Complex comprised of a VEGF Nucleic Acid Ligand and a Lipophilic Compound or Non-Immunogenic, High Molecular Weight Compound, wherein the Complex is further



associated with a Lipid Construct and the VEGF Nucleic Acid Ligand is further associated with the exterior of the Lipid Construct.

These and other objects, as well as the nature, scope and utilization of this invention, will become readily apparent to those skilled in the art from the following description and the appended claims.

### **BRIEF DESCRIPTION OF THE FIGURES**

Figures 1A-1Q show the molecular descriptions of NX213 (Figure 1A), NX278 (Figure 1B), scNX278 (Figure 1C), scNX213 (Figure 1D), NX31838-PL (Figure 1E), NX31838 Lipid Amide 1 (Figure 1F), NX31838 Lipid Amide 2 (Figure 1G), NX31838-40K PEG (Figure 1H), NX31838-20K PEG (Figure 1I), NX31838 40K PEG dimer with no linker (NX31838d0) (Figure 1J), NX31838 40K dimer with one C5 linker (NX31838d1) (Figure 1K), NX31838 40K PEG dimer with two C5 linkers (NX31838d2) (Figure 1L), C-5 Aminolinker (Figure 1M), Glycerol Bisphosphate Linker (Figure 1N), 18 Atom Spacer Linker (Figure 1O), Aminotetraethylene Glycol Linker (Figure 1P), 3'3'-dT (Figure 1Q), and NX31917 (Figure 1R). The 5' phosphate group of the ligand is depicted in the figures. mPEG stands for methyl polyethylene glycol. A lower case letter preceding a nucleotide indicates the following: m=2'-O-Methyl, a=2'-amino, r=ribo, and f=2'-fluoro. No letter preceding a nucleotide indicates a deoxyribonucleotide(2'H). 3'3'-dT indicates a 3'3' inverted phosphodiester linkage at the 3' end. An S following a nucleotide denotes a backbone modification consisting of a phosphorothioate internucleoside linkage.

Figure 2 shows binding properties of various Nucleic Acid Ligands to VEGF. The binding affinities of the unmodified Nucleic Acid Ligand (NX213, open circle), its dialkyl glycerol modified analog (NX278, open diamond) and liposomal NX278 (NX278-L, open square), along with the sequence scrambled (sc) controls (scNX213, closed circle;

scNX278, closed diamond; and scNX278-L, closed square) were determined by a competition electrophoretic mobility shift assay. NX213 is

5'-TsTsTsTs mAaCaC aCaUrG rAaUmG rGaUmA mGrAaC mGaCaC mGmGmG mGaUmG TsTsTsTsT-3' and scNX213 is

5 5'-TsTsTsTs mGaUaC mGmGaU mAaCrG mGrAmG aUmGrG rAaCnC mGaUaC mAaCmG TsTsTsTsT-3'

<sup>32</sup>P 5= end-labeled NX-213 (1.5 nM) was incubated in binding buffer (phosphate buffered saline with 0.01% human serum albumin) at 37 EC for 20 min in the presence of VEGF (0.33 nM) and competitor oligonucleotide (5 pM-0.33 :M). The <sup>32</sup>P NX-213/VEGF

10 complex was resolved from the free <sup>32</sup>P NX-213 by electrophoresis on 8% polyacrylamide gel (19:1 acrylamide:bis-acrylamide, Tris-borate, 89 mM, 1 mM EDTA as the running buffer). The intensity of the band corresponding to <sup>32</sup>P NX-213/VEGF complex at varying competitor concentrations was quantitated by phosphorimager analysis. Data normalized for the amount of complex formed in the absence of competitor were fitted by the least

15 squares method to the competition binding equation.

**Figure 3** shows the effect of various Nucleic Acid Ligands on VEGF-induced increases in vascular permeability. VEGF (20 nM) with or without Nucleic Acid Ligands was injected intradermally to guinea pigs that had previously received an injection of Evans blue dye. The amount of dye leakage was quantitated by measuring the relative amount of

20 light absorbed by the skin at the site of injection.

**Figure 4** shows that NX278-L inhibits KS cell growth. Growth of KSY-1 cells in the presence of various concentrations of NX213, NX278-L and scNX278 -L. KSY-1 cells were seeded in 24 well plates at a density of 1x10<sup>4</sup> cells/well on day 0. Fresh medium treated identically was replaced on days 1 and 3. The cell numbers were determined by

25 trypsinization of cells on day 5 or 6 of culture using particle coulter counter. The experiments were done in triplicates several times. Results shown are the average and SE of representative experiment.

**Figures 5A and 5B** show that NX278 inhibits KS cell growth in athymic mice. Athymic mice were implanted with KS tumor behind the forelegs on day 1. Mice were

30 treated with NX278-L (50 :g/day/mouse, **Figure 5A** and 150 :g/day/mouse, **Figure 5B**) by

intraperitoneal injection daily for five days beginning on day 2. Control mice were treated with empty liposomes using the same quantity of lipids as the Nucleic Acid Ligand treated group. The tumor sizes were measured over the period of two weeks. The tumors were removed on day 14 and measured.

5           **Figure 6** summarizes the data for the plasma concentration of NX31838 20K PEG ( $\square$ ), 40K PEG ( $\blacksquare$ ), and NX31838 (minus PEG) ( $\nabla$ ) as a function of time following the bolus injection.

**Figure 7** summarizes the data for the plasma concentration of NX31838 PL as a function of time following the bolus injection.

10           **Figures 8A-8D** shows changes in vascular permeability elicited by intradermal injection of VEGF protein (0.8 pmol)  $\pm$  Nucleic Acid Ligand/monoclonal antibody as indicated. Local extravasation of Evans blue dye was determined 30 min after injection by transillumination of harvested skin. Panels A, B, C, and D show the effect of co-mixing NX31838-20K PEG, NX31838-40K PEG, NX31838-PL, or NX31838d2-40K  
15 PEG with protein 30 min prior to injection. Values are mean  $\pm$  SEM. \*  $P < 0.05$  compared with VEGF alone. See **Figure 1** for molecular descriptions.

**Figures 9A-9C** shows the evaluation of Nucleic Acid Ligand attenuation of VEGF-induced corneal angiogenesis. Zero or three pmol of VEGF protein were incorporated in a biopolymer (Hydron) and implanted in the corneal stroma. Animals  
20 were treated intravenously twice daily with either PBS or Nucleic Acid Ligand as indicated for 5 days. Panels A, B, and C illustrate the effect of systemic treatment with NX31838-20K PEG, NX31838-40K PEG, or NX31838-PL Nucleic Acid Ligand on neovascularization. Values are mean  $\pm$  SEM. \*  $P < 0.05$  compared with 3 pmol VEGF + PBS group. See **Figure 1** for molecular descriptions.

25           **Figure 10** summarizes the data for the plasma ( $\circ, \Delta$ ) or vitreous ( $\bullet, \blacktriangle, \blacksquare$ ) concentration of NX31838-40K PEG as a function of time following administration.

**Figure 11** shows tumor growth curves of human A673 tumors growing subcutaneously (s.c.) in nude mice treated with 40 mg/kg or 10 mg/kg of VEGF NX31838 40K PEG Nucleic Acid Ligand (NX 31838 NAL) delivered twice a day (BID).  
30 A negative control consisted of a scrambled VEGF Nucleic Acid Ligand sequence,

NX31917 NAL (see **Figure 1R** for molecular description), dosed at 40 mg/kg twice daily, and a positive control consisted of an anti-VEGF monoclonal antibody mAb 26503.11 (R&D Systems) dosed at 100 µg/mouse twice weekly. Since there appeared to be no significant difference between the 40 mg/kg dose group and the 10 mg/kg dose group, no further dosing of the 40 mg/kg group occurred after day 14. Groups of 8 mice were implanted s.c. with  $1 \times 10^7$  A673 tumor cells on day 0, and treatment with test compounds by intraperitoneal injections initiated on day 1 for the duration of the experiment. Tumor volume, expressed as  $\text{mm}^3$ , was determined using the formula: Tumor vol. =  $L \times W^2/2$ .

**Figure 12** shows tumor growth curves of different dose schedules (comparison of twice daily dosing (BID) to once daily dosing (QD)), 40K PEG batches (comparison NX31838.07 batch with the new NX31838.04 batch), and different drug formulations (comparison of liposomal VEGF NX31838PL NAL to VEGF NX31838 NAL 40K PEG) of VEGF NX31838 Nucleic Acid Ligand (NAL). Groups of 8 mice were implanted s.c. with  $1 \times 10^7$  A673 tumor cells on day 0, and treatment with test compounds by intraperitoneal injections initiated on day 1 for the duration of the experiment. Several groups had animals where the tumors failed to grow, and consequently for final analysis some groups contain only 7 (NX31838.04 10 mg/kg BID, and NX31838.04 3 mg/kg BID), or 6 (NX31838.04 10 mg/kg QD, and NX31838.07 10 mg/kg BID) animals. Tumor volume, expressed as  $\text{mm}^3$ , was determined using the formula: Tumor vol. =  $L \times W^2/2$ .

**Figure 13** shows dose-dependent inhibition of A673 tumors growing subcutaneously (s.c.) in nude mice by VEGF NX31838 40K PEG Nucleic Acid Ligand (NX31838 NAL) delivered once daily. This titration failed to reach a no effect dose; tumor inhibition was still observed with the lowest (0.03 mg/kg) dose. Groups of 8 mice were implanted s.c. with  $1 \times 10^7$  A673 tumor cells on day 0, and treatment with test compounds by intraperitoneal injections initiated on day 1 for the duration of the experiment; group NX31838 NAL 3 mg/kg had 2 animals where tumors failed to grow and consequently contains only 6 animals. Tumor volume, expressed as  $\text{mm}^3$ , was determined using the formula: Tumor vol. =  $L \times W^2/2$ .

**Figure 14** shows tumor growth curves demonstrating inhibition of staged (i.e., established) A673 tumors growing subcutaneously (s.c.) in nude mice by VEGF NX31838 40K PEG Nucleic Acid Ligand (NAL) delivered once daily. A positive control consisted of an anti-VEGF monoclonal antibody mAb 26503.11 (R&D Systems) dosed at 100 µg/mouse twice weekly. Mice were implanted with  $1 \times 10^7$  A673 cells, and tumors allowed to grow to a volume of  $200 \pm 100 \text{ mm}^3$ , at which time animals were sorted by weight, tattooed for permanent identification, and treatment with test compounds by intraperitoneal injections initiated and continued for the duration of the experiment. Each point represents the mean of 8 mice. Tumor volume, expressed as  $\text{mm}^3$ , was determined using the formula: Tumor vol. =  $L \times W^2/2$ .

**Figure 15** summarizes the data for the plasma concentration of NX213, NX278, NX278-Liposome following bolus injection.

**Figure 16** shows the growth curves of KSY-1 tumors implanted subcutaneously in nude mice. The mice were treated by intraperitoneal injections of NX31917 40K PEG or NX31838 40K PEG (30 mg/kg) or PBS twice daily for the duration of the experiment. Treatment was initiated one day after subcutaneous implantation of  $2 \times 10^7$  KSY-1 cells in the hind flank of nude mice. Four mice were used in each group. Errors are SEM.

## **DETAILED DESCRIPTION OF THE INVENTION**

### **DEFINITIONS:**

**"Covalent Bond"** is the chemical bond formed by the sharing of electrons.

**"Non-Covalent Interactions"** are means by which molecular entities are held together by interactions other than Covalent Bonds including ionic interactions and hydrogen bonds.

**"Lipophilic Compounds"** are compounds which have the propensity to associate with or partition into lipid and/or other materials or phases with low dielectric constants, including structures that are comprised substantially of lipophilic components. Lipophilic Compounds include lipids as well as non-lipid containing compounds that have the propensity to associate with lipid (and/or other materials or phases with low dielectric constants). Cholesterol, phospholipid, and glycerolipids, such as dialkylglycerol, and

diacylglycerol, and glycerol amide lipids are further examples of Lipophilic Compounds. In one preferred embodiment of the invention, the lipophilic compound covalently linked to the VEGF Nucleic Acid Ligand is a glycerolipid having the structure



where  $\text{R}^1$ ,  $\text{R}^2$ , and  $\text{R}^3$  are independently selected from the group consisting of  $\text{CH}_3(\text{CH}_2)_n\text{-O(PO}_3\text{)-CH}_2\text{-}$ ; and  $\text{CH}_3(\text{CH}_2)_n\text{-CONH}_2\text{-CH}_2\text{-}$ ,  $\text{CH}_3(\text{CH}_2)_n\text{O-}$ ,  $\text{CH}_3(\text{CH}_2)_n\text{OCH}_2\text{-}$ ,  $\text{CH}_3(\text{CH}_2)_n(\text{CO})\text{OCH}_2\text{-}$ ,  $\text{CH}_3(\text{CH}_2)_n(\text{CO})\text{O-}$  and  $\text{X-}$ , wherein at least one must be  $\text{X-}$ , and  $\text{X}$  is independently selected from the group consisting of  $(\text{PO}_4)$ ,  $\text{O}$  and  $\text{CH}_2\text{OC=O}$ , and wherein  $n=0\text{-}30$ , preferably  $10\text{-}20$ . When  $\text{R}$  is  $\text{CH}_3(\text{CH}_2)_n\text{-O(PO}_3\text{)-CH}_2\text{-}$ , the Lipophilic Compound is a phospholipid. When  $\text{R}$  is  $\text{CH}_3(\text{CH}_2)_n\text{-CONH}_2\text{-CH}_2\text{-}$ , the Lipophilic Compound is a glycerol amide lipid. When  $\text{R}$  is  $\text{CH}_3(\text{CH}_2)_n\text{O-}$  or  $\text{CH}_3(\text{CH}_2)_n\text{OCH}_2\text{-}$ , the Lipophilic Compound is a dialkylglycerol lipid. When  $\text{R}$  is  $\text{CH}_3(\text{CH}_2)_n(\text{CO})\text{OCH}_2\text{-}$  or  $\text{CH}_3(\text{CH}_2)_n(\text{CO})\text{O-}$ ; the Lipophilic Compound is diacylglycerol lipid. In a preferred embodiment,  $\text{R}^3$  is  $\text{X-}$ .

"Complex" as used herein describes the molecular entity formed by the covalent linking of a VEGF Nucleic Acid Ligand to a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound. In certain embodiments of the present invention, the Complex is depicted as  $\text{A-B-Y}$ , wherein  $\text{A}$  is a Lipophilic Compound or Non-Immunogenic, High Molecular Weight Compound as described herein;  $\text{B}$  is optional, and may be one or more linkers  $\text{Z}$ ; and  $\text{Y}$  is a VEGF Nucleic Acid Ligand.

"Lipid Constructs," for purposes of this invention, are structures containing lipids, phospholipids, or derivatives thereof comprising a variety of different structural arrangements which lipids are known to adopt in aqueous suspension. These structures include, but are not limited to, Lipid Bilayer Vesicles, micelles, Liposomes, emulsions, lipid ribbons or sheets, and may be complexed with a variety of drugs and components which are known to be pharmaceutically acceptable. In the preferred embodiment, the Lipid Construct is a Liposome. The preferred Liposome is unilamellar and has a relative size less than  $200\text{ nm}$ . Common additional components in Lipid Constructs include cholesterol and  $\alpha$ -

tocopherol, among others. The Lipid Constructs may be used alone or in any combination which one skilled in the art would appreciate to provide the characteristics desired for a particular application. In addition, the technical aspects of Lipid Constructs and Liposome formation are well known in the art and any of the methods commonly practiced in the field  
5 may be used for the present invention.

**"Nucleic Acid Ligand"** as used herein is a non-naturally occurring Nucleic Acid having a desirable action on a Target. The Target of the present invention is VEGF, hence the term VEGF Nucleic Acid Ligand. A desirable action includes, but is not limited to, binding of the Target, catalytically changing the Target, reacting with the Target in a way  
10 which modifies/alters the Target or the functional activity of the Target, covalently attaching to the Target as in a suicide inhibitor, facilitating the reaction between the Target and another molecule. In the preferred embodiment, the action is specific binding affinity for VEGF, wherein the Nucleic Acid Ligand is not a Nucleic Acid having the known physiological function of being bound by VEGF.

15 In preferred embodiments of the invention, the VEGF Nucleic Acid Ligand of the Complexes and Lipid Constructs of the invention are identified by the SELEX methodology. VEGF Nucleic Acid Ligands are identified from a Candidate Mixture of Nucleic Acids, said Nucleic Acid being a ligand of VEGF, by the method comprising a) contacting the Candidate Mixture with VEGF, wherein Nucleic Acids having an increased  
20 affinity to VEGF relative to the Candidate Mixture may be partitioned from the remainder of the Candidate Mixture; b) partitioning the increased affinity Nucleic Acids from the remainder of the Candidate Mixture; and c) amplifying the increased affinity Nucleic Acids to yield a ligand-enriched mixture of Nucleic Acids (see United States Patent Application Serial No. 08/233,012, filed April 25, 1994, entitled "High Affinity Oligonucleotides to  
25 Vascular Endothelial Growth Factor (VEGF)," United States Patent Application Serial No. 08/447,169, filed May 19, 1995, entitled "High Affinity Oligonucleotide Ligands to Vascular Endothelial Growth Factor (VEGF)," which are hereby incorporated by reference herein).

**"Candidate Mixture"** is a mixture of Nucleic Acids of differing sequence from  
30 which to select a desired ligand. The source of a Candidate Mixture can be from naturally-

occurring Nucleic Acids or fragments thereof, chemically synthesized Nucleic Acids, enzymatically synthesized Nucleic Acids or Nucleic Acids made by a combination of the foregoing techniques. In a preferred embodiment, each Nucleic Acid has fixed sequences surrounding a randomized region to facilitate the amplification process.

5           **"Nucleic Acid"** means either DNA, RNA, single-stranded or double-stranded and any chemical modifications thereof. Modifications include, but are not limited to, those which provide other chemical groups that incorporate additional charge, polarizability, hydrogen bonding, electrostatic interaction, and fluxionality to the Nucleic Acid Ligand bases or to the Nucleic Acid Ligand as a whole. Such modifications include, but are not  
10   limited to, 2'-position sugar modifications, 5-position pyrimidine modifications, 8-position purine modifications, modifications at exocyclic amines, substitution of 4-thiouridine, substitution of 5-bromo or 5-iodo-uracil, backbone modifications such as internucleoside phosphorothioate linkages, methylations, unusual base-pairing combinations such as the isobases isocytidine and isoguanidine and the like. Modifications can also include 3' and 5'  
15   modifications such as capping.

**"Non-Immunogenic, High Molecular Weight Compound"** is a compound between approximately 1000 Da to 1,000,000 Da, more preferably approximately 1000 Da to 500,000 Da, and most preferably approximately 1000 Da to 200,000 Da, that typically does not generate an immunogenic response. For the purposes of this invention, an  
20   immunogenic response is one that causes the organism to make antibody proteins. Examples of Non-Immunogenic, High Molecular Weight Compounds include Polyalkylene Glycol and polyethylene glycol. In one preferred embodiment of the invention, the Non-Immunogenic, High Molecular Weight Compound covalently linked to the VEGF Nucleic Acid Ligand is a polyalkylene glycol and has the structure  $R(O(CH_2)_x)_nO-$ , where R is  
25   independently selected from the group consisting of H and  $CH_3$ ,  $x=2-5$ , and  $n \approx MW$  of the Polyalkylene Glycol/16 + 14x. In the preferred embodiment of the present invention, the molecular weight is about between 10-80kDa. In the most preferred embodiment, the molecular weight of the polyalkylene glycol is about between 20-45kDa. In the most preferred embodiment,  $x=2$  and  $n=9 \times 10^2$ . There can be one or more Polyalkylene Glycols



attached to the same VEGF Nucleic Acid Ligand, with the sum of the molecular weights preferably being between 10-80kDa, more preferably 20-45kDa.

In certain embodiments, the Non-Immunogenic, High Molecular Weight Compound can also be a Nucleic Acid Ligand.

- 5           **"Lipid Bilayer Vesicles"** are closed, fluid-filled microscopic spheres which are formed principally from individual molecules having polar (hydrophilic) and non-polar (lipophilic) portions. The hydrophilic portions may comprise phosphato, glycerylphosphato, carboxy, sulfato, amino, hydroxy, choline and other polar groups. Examples of non-polar groups are saturated or unsaturated hydrocarbons such as alkyl, alkenyl or other lipid groups. Sterols (e.g., cholesterol) and other pharmaceutically acceptable components (including anti-oxidants like alpha-tocopherol) may also be included to improve vesicle stability or confer other desirable characteristics.

- "Liposomes"** are a subset of Lipid Bilayer Vesicles and are comprised principally of phospholipid molecules which contain two hydrophobic tails consisting of long fatty acid chains. Upon exposure to water, these molecules spontaneously align to form a bilayer membrane with the lipophilic ends of the molecules in each layer associated in the center of the membrane and the opposing polar ends forming the respective inner and outer surface of the bilayer membrane. Thus, each side of the membrane presents a hydrophilic surface while the interior of the membrane comprises a lipophilic medium. These membranes when formed are generally arranged in a system of concentric closed membranes separated by interlamellar aqueous phases, in a manner not dissimilar to the layers of an onion, around an internal aqueous space. These multilamellar vesicles (MLV) can be converted into unilamellar vesicles (UV), with the application of a shearing force.

- "Cationic Liposome"** is a Liposome that contains lipid components that have an overall positive charge at physiological pH.

- "SELEX"** methodology involves the combination of selection of Nucleic Acid Ligands which interact with a Target in a desirable manner, for example binding to a protein, with amplification of those selected Nucleic Acids. Iterative cycling of the selection/amplification steps allows selection of one or a small number of Nucleic Acids which interact most strongly with the Target from a pool which contains a very large

number of Nucleic Acids. Cycling of the selection/amplification procedure is continued until a selected goal is achieved. The SELEX methodology is described in the SELEX Patent Applications.

5       **"Target"** means any compound or molecule of interest for which a ligand is desired. A Target can be a protein (such as VEGF, thrombin, and selectin), peptide, carbohydrate, polysaccharide, glycoprotein, hormone, receptor, antigen, antibody, virus, substrate, metabolite, transition state analog, cofactor, inhibitor, drug, dye, nutrient, growth factor, etc. without limitation. The principal Target of the subject invention is VEGF.

10       **"Improved Pharmacokinetic Properties"** means that the VEGF Nucleic Acid Ligand covalently linked to a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound or in association with a Lipid Construct shows a longer circulation half-life *in vivo* relative to the same VEGF Nucleic Acid Ligand not in association with a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound or in association with a Lipid Construct.

15       **"Linker"** is a molecular entity that connects two or more molecular entities through Covalent Bond or Non-Covalent Interactions, and can allow spatial separation of the molecular entities in a manner that preserves the functional properties of one or more of the molecular entities. A linker can also be known as a spacer. Examples of Linkers, include but are not limited to, the structures shown in **Figures 1M-1P**.

20       **"Therapeutic"** as used herein, includes treatment and/or prophylaxis. When used, Therapeutic refers to humans and other animals.

      This invention includes RNA ligands to VEGF that are comprised of 2'F-modified nucleotides. This invention further includes the specific RNA ligands to VEGF shown in  
25   **Tables 2-6** (SEQ ID NOS: ). More specifically, this invention includes nucleic acid sequences that are substantially homologous to and that have substantially the same ability to bind VEGF as the specific nucleic acid ligands shown in **Tables 2-6**. By substantially homologous it is meant a degree of primary sequence homology in excess of 70%, most preferably in excess of 80%, and even more preferably in excess of 90%, 95%, or 99%.  
30   The percentage of homology as described herein is calculated as the percentage of

nucleotides found in the smaller of the two sequences which align with identical nucleotide residues in the sequence being compared when 1 gap in a length of 10 nucleotides may be introduced to assist in that alignment. Substantially the same ability to bind VEGF means that the affinity is within one or two orders of magnitude of the affinity of the ligands described herein. It is well within the skill of those of ordinary skill in the art to determine whether a given sequence – substantially homologous to those specifically described herein – has the same ability to bind VEGF.

A review of the sequence homologies of the nucleic acid ligands of VEGF shown in **Tables 2-6** (SEQ ID NOS: ) shows that sequences with little or no primary homology may have substantially the same ability to bind VEGF. For these reasons, this invention also includes Nucleic Acid Ligands that have substantially the same postulated structure or structural motifs and ability to bind VEGF as the nucleic acid ligands shown in **Tables 2-6**. Substantially the same structure or structural motifs can be postulated by sequence alignment using the Zukerfold program (see Zuker (1989) Science 244:48-52). As would be known in the art, other computer programs can be used for predicting secondary structure and structural motifs. Substantially the same structure or structural motif of Nucleic Acid Ligands in solution or as a bound structure can also be postulated using NMR or other techniques as would be known in the art.

Further included in this invention is a method for preparing a Complex comprised of a VEGF Nucleic Acid Ligand and a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound by the method comprising identifying a Nucleic Acid Ligand from a Candidate Mixture of Nucleic Acids where the Nucleic Acid is a ligand of VEGF by the method of (a) contacting the Candidate Mixture of Nucleic Acids with VEGF, (b) partitioning between members of said Candidate Mixture on the basis of affinity to VEGF, and c) amplifying the selected molecules to yield a mixture of Nucleic Acids enriched for Nucleic Acid sequences with a relatively higher affinity for binding to VEGF, and covalently linking said identified VEGF Nucleic Acid Ligand with a Non-Immunogenic, High Molecular Weight Compound or a Lipophilic Compound.

It is a further object of the present invention to provide Complexes comprising one or more VEGF Nucleic Acid Ligands covalently linked to a Non-Immunogenic, High

Molecular Weight Compound or Lipophilic Compound. Such Complexes have one or more of the following advantages over a VEGF Nucleic Acid Ligand not in association with a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound:

- 1) Improved Pharmacokinetic Properties, and 2) improved capacity for intracellular delivery, or 3) improved capacity for targeting. Complexes further associated with a Lipid Construct have the same advantages.

The Complexes or the Lipid Constructs comprising the VEGF Nucleic Acid Ligand or Complexes may benefit from one, two, or three of these advantages. For example, a Lipid Construct of the present invention may be comprised of a) a Liposome, b) a drug that is encapsulated within the interior of the Liposome, and c) a Complex comprised of a VEGF Nucleic Acid Ligand and Lipophilic Compound, wherein the VEGF Nucleic Acid Ligand component of the Complex is associated with and projecting from the exterior of the Lipid Construct. In such a case, the Lipid Construct comprising a Complex will 1) have Improved Pharmacokinetic Properties, 2) have enhanced capacity for intracellular delivery of the encapsulated drug, and 3) be specifically targeted to the preselected location in vivo that is expressing VEGF by the exteriorly associated VEGF Nucleic Acid Ligand.

In another embodiment, this invention provides a method for improving the pharmacokinetic properties of a VEGF Nucleic Acid Ligand by covalently linking the VEGF Nucleic Acid Ligand with a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound to form a Complex and administering the Complex to a patient. The invention further relates to a method for improving the pharmacokinetic properties of a VEGF Nucleic Acid Ligand by further associating the Complex with a Lipid Construct.

In another embodiment, the Complex of the present invention is comprised of a VEGF Nucleic Acid Ligand covalently attached to a Lipophilic Compound, such as a glycerolipid, or a Non-Immunogenic, High Molecular Weight Compound, such as Polyalkylene Glycol or polyethylene glycol (PEG). In these cases, the pharmacokinetic properties of the Complex will be enhanced relative to the VEGF Nucleic Acid Ligand alone. In another embodiment, the pharmacokinetic properties of the VEGF Nucleic Acid Ligand is enhanced relative to the VEGF Nucleic Acid Ligand alone when the VEGF Nucleic Acid Ligand is covalently attached to a Non-Immunogenic, High

Molecular Weight Compound or Lipophilic Compound and is further associated with a Lipid Construct or the VEGF Nucleic Acid Ligand is encapsulated within a Lipid Construct.

In embodiments where there are multiple VEGF Nucleic Acid Ligands, there is an increase in avidity due to multiple binding interactions with VEGF. Furthermore, in  
5    embodiments where the Complex is comprised of multiple VEGF Nucleic Acid Ligands, the pharmacokinetic properties of the Complex will be improved relative to one VEGF Nucleic Acid Ligand alone. In embodiments where a Lipid Construct comprises multiple Nucleic Acid Ligands or Complexes, the Pharmacokinetic Properties of the VEGF Nucleic  
10   Acid Ligand may be improved relative to Lipid Constructs in which there is only one Nucleic Acid Ligand or Complex.

In certain embodiments of the invention, the Complex of the present invention is comprised of a VEGF Nucleic Acid Ligand attached to one (dimeric) or more (multimeric) other Nucleic Acid Ligands. The Nucleic Acid Ligand can be to VEGF or a  
15   different Target. In embodiments where there are multiple VEGF Nucleic Acid Ligands, there is an increase in avidity due to multiple binding interactions with VEGF. Furthermore, in embodiments of the invention where the Complex is comprised of a VEGF Nucleic Acid Ligand attached to one or more other VEGF Nucleic Acid Ligands, the pharmacokinetic properties of the Complex will be improved relative to one VEGF  
20   Nucleic Acid Ligand alone.

The Non-Immunogenic, High Molecular Weight compound or Lipophilic Compound may be covalently bound to a variety of positions on the VEGF Nucleic Acid Ligand, such as to an exocyclic amino group on the base, the 5-position of a pyrimidine nucleotide, the 8-position of a purine nucleotide, the hydroxyl group of the phosphate, or a  
25   hydroxyl group or other group at the 5' or 3' terminus of the VEGF Nucleic Acid Ligand. In embodiments where the Lipophilic Compound is a glycerolipid, or the Non-Immunogenic, High Molecular Weight Compound is polyalkylene glycol or polyethylene glycol, preferably it is bonded to the 5' or 3' hydroxyl of the phosphate group thereof. In the most preferred embodiment, the Lipophilic Compound or Non-Immunogenic, High Molecular  
30   Weight Compound is bonded to the 5' hydroxyl of the phosphate group of the Nucleic Acid

Ligand. Attachment of the Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound to the VEGF Nucleic Acid Ligand can be done directly or with the utilization of Linkers or Spacers. In embodiments where the Lipid Construct comprises a Complex, or where the VEGF Nucleic Acid Ligands are encapsulated within the Liposome, a Non-Covalent Interaction between the VEGF Nucleic Acid Ligand or the Complex and the Lipid Construct is preferred.

One problem encountered in the therapeutic use of Nucleic Acids is that oligonucleotides in their phosphodiester form may be quickly degraded in body fluids by intracellular and extracellular enzymes such as endonucleases and exonucleases before the desired effect is manifest. Certain chemical modifications of the VEGF Nucleic Acid Ligand can be made to increase the *in vivo* stability of the VEGF Nucleic Acid Ligand or to enhance or to mediate the delivery of the VEGF Nucleic Acid Ligand. Modifications of the VEGF Nucleic Acid Ligands contemplated in this invention include, but are not limited to, those which provide other chemical groups that incorporate additional charge, polarizability, hydrophobicity, hydrogen bonding, electrostatic interaction, and fluxionality to the VEGF Nucleic Acid Ligand bases or to the VEGF Nucleic Acid Ligand as a whole. Such modifications include, but are not limited to, 2'-position sugar modifications, 5-position pyrimidine modifications, 8-position purine modifications, modifications at exocyclic amines, substitution of 4-thiouridine, substitution of 5-bromo or 5-iodo-uracil; backbone modifications, phosphorothioate or alkyl phosphate modifications, methylations, unusual base-pairing combinations such as the isobases isocytidine and isoguanidine and the like. Modifications can also include 3' and 5' modifications such as capping.

Where the Nucleic Acid Ligands are derived by the SELEX method, the modifications can be pre- or post- SELEX modifications. Pre-SELEX modifications yield VEGF Nucleic Acid Ligands with both specificity for VEGF and improved *in vivo* stability. Post-SELEX modifications made to 2'-OH Nucleic Acid Ligands can result in improved *in vivo* stability without adversely affecting the binding capacity of the Nucleic Acid Ligands. The preferred modifications of the VEGF Nucleic Acid Ligands of the subject invention are 5' and 3' phosphorothioate capping and/or 3'3' inverted phosphodiester linkage at the 3' end. In the most preferred embodiment, the preferred modification of the VEGF Nucleic Acid

Ligand is 3'3' inverted phosphodiester linkage at the 3' end. Additional 2' fluoro (2'-F), 2' amino (2'-NH<sub>2</sub>) and 2' O methyl (2'-OMe) modification of some or all of the nucleotides is preferred.

In another aspect of the present invention, the covalent linking of the VEGF Nucleic  
5 Acid Ligand with a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound results in Improved Pharmacokinetic Properties (i.e., slower clearance rate) relative to the VEGF Nucleic Acid Ligand not in association with a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound.

In another aspect of the present invention, the Complex comprising a VEGF Nucleic  
10 Acid Ligand and Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound can be further associated with a Lipid Construct. This association may result in Improved Pharmacokinetic Properties relative to the VEGF Nucleic Acid Ligand or Complex not in association with a Lipid Construct. The VEGF Nucleic Acid Ligand or Complex can be associated with the Lipid Construct through covalent or Non-Covalent  
15 Interactions. In another aspect, the VEGF Nucleic Acid Ligand can be associated with the Lipid Construct through Covalent or Non-Covalent Interactions. In a preferred embodiment, the association is through Non-Covalent Interactions. In a preferred embodiment, the Lipid Construct is a Lipid Bilayer Vesicle. In the most preferred embodiment, the Lipid Construct is a Liposome.

20 Liposomes for use in the present invention can be prepared by any of the various techniques presently known in the art or subsequently developed. Typically, they are prepared from a phospholipid, for example, distearoyl phosphatidylcholine, and may include other materials such as neutral lipids, for example, cholesterol, and also surface modifiers such as positively charged (e.g., sterylamine or aminomannose or aminomannitol  
25 derivatives of cholesterol) or negatively charged (e.g., dicetyl phosphate, phosphatidyl glycerol) compounds. Multilamellar Liposomes can be formed by conventional techniques, that is, by depositing a selected lipid on the inside wall of a suitable container or vessel by dissolving the lipid in an appropriate solvent, and then evaporating the solvent to leave a thin film on the inside of the vessel or by spray drying. An aqueous phase is then added to  
30 the vessel with a swirling or vortexing motion which results in the formation of MLVs.

UVs can then be formed by homogenization, sonication or extrusion (through filters) of MLV's. In addition, UVs can be formed by detergent removal techniques.

In certain embodiments of this invention, the Lipid Construct comprises a targeting VEGF Nucleic Acid Ligand(s) associated with the surface of the Lipid Construct and an encapsulated therapeutic or diagnostic agent. Preferably the Lipid Construct is a Liposome. Preformed Liposomes can be modified to associate with the VEGF Nucleic Acid Ligands. For example, a Cationic Liposome associates through electrostatic interactions with the VEGF Nucleic Acid Ligand. A VEGF Nucleic Acid Ligand covalently linked to a Lipophilic Compound, such as a glycerolipid, can be added to preformed Liposomes whereby the glyccrolipid, phospholipid, or glycerol amide lipid becomes associated with the liposomal membrane. Alternatively, the VEGF Nucleic Acid Ligand can be associated with the Liposome during the formulation of the Liposome.

It is well known in the art that Liposomes are advantageous for encapsulating or incorporating a wide variety of therapeutic and diagnostic agents. Any variety of compounds can be enclosed in the internal aqueous compartment of the Liposomes. Illustrative therapeutic agents include antibiotics, antiviral nucleosides, antifungal nucleosides, metabolic regulators, immune modulators, chemotherapeutic drugs, toxin antidotes, DNA, RNA, antisense oligonucleotides, etc. By the same token, the Lipid Bilayer Vesicles may be loaded with a diagnostic radionuclide (e.g., Indium 111, Iodine 131, Yttrium 90, Phosphorous 32, or gadolinium) and fluorescent materials or other materials that are detectable in *in vitro* and *in vivo* applications. It is to be understood that the therapeutic or diagnostic agent can be encapsulated by the Liposome walls in the aqueous interior. Alternatively, the carried agent can be a part of, that is, dispersed or dissolved in the vesicle wall-forming materials.

During Liposome formation, water soluble carrier agents may be encapsulated in the aqueous interior by including them in the hydrating solution, and lipophilic molecules incorporated into the lipid bilayer by inclusion in the lipid formulation. In the case of certain molecules (e.g., cationic or anionic lipophilic drugs), loading of the drug into preformed Liposomes may be accomplished, for example, by the methods described



in U.S. Patent No. 4,946,683, the disclosure of which is incorporated herein by reference. Following drug encapsulation, the Liposomes are processed to remove unencapsulated drug through processes such as gel chromatography or ultrafiltration. The Liposomes are then typically sterile filtered to remove any microorganisms which may be present in the  
5 suspension. Microorganisms may also be removed through aseptic processing.

If one wishes to encapsulate large hydrophilic molecules with Liposomes, larger unilamellar vesicles can be formed by methods such as the reverse-phase evaporation (REV) or solvent infusion methods. Other standard methods for the formation of Liposomes are known in the art, for example, methods for the commercial  
10 production of Liposomes include the homogenization procedure described in U.S. Patent No. 4,753,788 and the thin-film evaporation method described in U.S. Patent No. 4,935,171, which are incorporated herein by reference.

It is to be understood that the therapeutic or diagnostic agent can also be associated with the surface of the Lipid Bilayer Vesicle. For example, a drug can be  
15 attached to a phospholipid or glyceride (a prodrug). The phospholipid or glyceride portion of the prodrug can be incorporated into the lipid bilayer of the Liposome by inclusion in the lipid formulation or loading into preformed Liposomes (see U.S. Patent Nos 5,194,654 and 5,223,263, which are incorporated by reference herein).

It is readily apparent to one skilled in the art that the particular Liposome  
20 preparation method will depend on the intended use and the type of lipids used to form the bilayer membrane.

Lee and Low (1994, JBC, 269: 3198-3204) and DeFrees et al. (1996, JACS, 118: 6101-6104) first showed that co-formulation of ligand-PEG-lipid with lipid components gave liposomes with both inward and outward facing orientations of the PEG-ligand.  
25 Passive anchoring was outlined by Zalipsky et al. (1997, Bioconj. Chem. 8: 111-118) as a method for anchoring oligopeptide and oligosaccharide ligands exclusively to the external surface of liposomes. The central concept presented in their work is that ligand-PEG-lipid conjugates can be prepared and then formulated into pre-formed liposomes via spontaneous incorporation ("anchoring") of the lipid tail into the existing lipid bilayer.  
30 The lipid group undergoes this insertion in order to reach a lower free energy state via the

removal of its hydrophobic lipid anchor from aqueous solution and its subsequent positioning in the hydrophobic lipid bilayer. The key advantage to such a system is that the oligo-lipid is anchored exclusively to the exterior of the lipid bilayer. Thus, no oligo-lipids are wasted by being unavailable for interactions with their biological targets by  
5 being in an inward-facing orientation.

The efficiency of delivery of a VEGF Nucleic Acid Ligand to cells may be optimized by using lipid formulations and conditions known to enhance fusion of Liposomes with cellular membranes. For example, certain negatively charged lipids such as phosphatidylglycerol and phosphatidylserine promote fusion, especially in the  
10 presence of other fusogens (e.g., multivalent cations like  $\text{Ca}^{2+}$ , free fatty acids, viral fusion proteins, short chain PEG, lysolecithin, detergents and surfactants). Phosphatidylethanolamine may also be included in the Liposome formulation to increase membrane fusion and, concomitantly, enhance cellular delivery. In addition, free fatty acids and derivatives thereof, containing, for example, carboxylate moieties, may be used  
15 to prepare pH-sensitive Liposomes which are negatively charged at higher pH and neutral or protonated at lower pH. Such pH-sensitive Liposomes are known to possess a greater tendency to fuse.

In the preferred embodiment, the VEGF Nucleic Acid Ligands of the present invention are derived from the SELEX methodology. SELEX is described in U.S. Patent  
20 Application Serial No. 07/536,428, entitled Systematic Evolution of Ligands by Exponential Enrichment, now abandoned, U.S. Patent Application Serial No. 07/714,131, filed June 10, 1991, entitled Nucleic Acid Ligands, now United States Patent No. 5,475,096, United States Patent Application Serial No. 07/931,473, filed August 17, 1992, entitled Nucleic Acid Ligands, now United States Patent No. 5,270,163 (see also WO  
25 91/19813). These applications, each specifically incorporated herein by reference, are collectively called the SELEX Patent Applications.

The SELEX process provides a class of products which are Nucleic Acid molecules, each having a unique sequence, and each of which has the property of binding specifically to a desired Target compound or molecule. Target molecules are preferably proteins, but  
30 can also include among others carbohydrates, peptidoglycans and a variety of small

molecules. SELEX methodology can also be used to Target biological structures, such as cell surfaces or viruses, through specific interaction with a molecule that is an integral part of that biological structure.

In its most basic form, the SELEX process may be defined by the following series  
5 of steps:

1) A Candidate Mixture of Nucleic Acids of differing sequence is prepared. The Candidate Mixture generally includes regions of fixed sequences (i.e., each of the members of the Candidate Mixture contains the same sequences in the same location) and regions of randomized sequences. The fixed sequence regions are selected either: (a) to assist in the  
10 amplification steps described below, (b) to mimic a sequence known to bind to the Target, or (c) to enhance the concentration of a given structural arrangement of the Nucleic Acids in the Candidate Mixture. The randomized sequences can be totally randomized (i.e., the probability of finding a base at any position being one in four) or only partially randomized (e.g., the probability of finding a base at any location can be selected at any level between 0  
15 and 100 percent).

2) The Candidate Mixture is contacted with the selected Target under conditions favorable for binding between the Target and members of the Candidate Mixture. Under these circumstances, the interaction between the Target and the Nucleic Acids of the Candidate Mixture can be considered as forming Nucleic Acid-target pairs between the  
20 Target and those Nucleic Acids having the strongest affinity for the Target.

3) The Nucleic Acids with the highest affinity for the target are partitioned from those Nucleic Acids with lesser affinity to the target. Because only an extremely small number of sequences (and possibly only one molecule of Nucleic Acid) corresponding to the highest affinity Nucleic Acids exist in the Candidate Mixture, it is generally desirable to  
25 set the partitioning criteria so that a significant amount of the Nucleic Acids in the Candidate Mixture (approximately 5-50%) are retained during partitioning.

4) Those Nucleic Acids selected during partitioning as having the relatively higher affinity for the target are then amplified to create a new Candidate Mixture that is enriched in Nucleic Acids having a relatively higher affinity for the target.

5) By repeating the partitioning and amplifying steps above, the newly formed Candidate Mixture contains fewer and fewer unique sequences, and the average degree of affinity of the Nucleic Acids to the target will generally increase. Taken to its extreme, the SELEX process will yield a Candidate Mixture containing one or a small number of unique Nucleic Acids representing those Nucleic Acids from the original Candidate Mixture having the highest affinity to the target molecule.

The basic SELEX method has been modified to achieve a number of specific objectives. For example, United States Patent Application Serial No. 07/960,093, filed October 14, 1992, entitled "Method for Selecting Nucleic Acids on the Basis of Structure," describes the use of SELEX in conjunction with gel electrophoresis to select Nucleic Acid molecules with specific structural characteristics, such as bent DNA. United States Patent Application Serial No. 08/123,935, filed September 17, 1993, entitled "Photoselection of Nucleic Acid Ligands," describes a SELEX based method for selecting Nucleic Acid Ligands containing photoreactive groups capable of binding and/or photocrosslinking to and/or photoinactivating a target molecule. United States Patent Application Serial No. 08/134,028, filed October 7, 1993, entitled "High-Affinity Nucleic Acid Ligands That Discriminate Between Theophylline and Caffeine," now United States Patent No. 5,580,737, describes a method for identifying highly specific Nucleic Acid Ligands able to discriminate between closely related molecules, termed Counter-SELEX. United States Patent Application Serial No. 08/143,564, filed October 25, 1993, entitled "Systematic Evolution of Ligands by EXponential Enrichment: Solution SELEX," now United States Patent No. 5,567,588, describes a SELEX-based method which achieves highly efficient partitioning between oligonucleotides having high and low affinity for a target molecule. United States Patent Application Serial No. 07/964,624, filed October 21, 1992, entitled "Methods of Producing Nucleic Acid Ligands," now United States Patent No. 5,496,938, describes methods for obtaining improved Nucleic Acid Ligands after SELEX has been performed. United States Patent Application Serial No. 08/400,440, filed March 8, 1995, entitled "Systematic Evolution of Ligands by EXponential Enrichment: Chemi-SELEX," describes methods for covalently linking a ligand to its target.

The SELEX method encompasses the identification of high-affinity Nucleic Acid Ligands containing modified nucleotides conferring improved characteristics on the ligand, such as improved *in vivo* stability or improved delivery characteristics. Examples of such modifications include chemical substitutions at the ribose and/or phosphate and/or base positions. SELEX-identified Nucleic Acid Ligands containing modified nucleotides are described in United States Patent Application Serial No. 08/117,991, filed September 8, 1993, entitled "High Affinity Nucleic Acid Ligands Containing Modified Nucleotides," now United States Patent No. 5,660,985, that describes oligonucleotides containing nucleotide derivatives chemically modified at the 5- and 2'-positions of pyrimidines.

United States Patent Application Serial No. 08/134,028, *supra*, describes highly specific Nucleic Acid Ligands containing one or more nucleotides modified with 2'-amino (2'-NH<sub>2</sub>), 2'-fluoro (2'-F), and/or 2'-O-methyl (2'-OMe). United States Patent Application Serial No. 08/264,029, filed June 22, 1994, entitled "Novel Method of Preparation of 2' Modified Pyrimidine Intramolecular Nucleophilic Displacement," describes oligonucleotides containing various 2'-modified pyrimidines.

The SELEX method encompasses combining selected oligonucleotides with other selected oligonucleotides and non-oligonucleotide functional units as described in United States Patent Application Serial No. 08/284,063, filed August 2, 1994, entitled "Systematic Evolution of Ligands by Exponential Enrichment: Chimeric SELEX," now United States Patent No. 5,637,459, and United States Patent Application Serial No. 08/234,997, filed April 28, 1994, entitled "Systematic Evolution of Ligands by Exponential Enrichment: Blended SELEX," respectively. These applications allow the combination of the broad array of shapes and other properties, and the efficient amplification and replication properties, of oligonucleotides with the desirable properties of other molecules.

The SELEX method further encompasses combining selected Nucleic Acid Ligands with Lipophilic Compounds or Non-Immunogenic, High Molecular Weight Compounds in a diagnostic or therapeutic Complex as described in United States Patent Application Serial No. 08/434,465, filed May 4, 1995, entitled "Nucleic Acid Complexes." The SELEX method further encompasses combining selected VEGF Nucleic Acid Ligands with lipophilic compounds, such as diacyl glycerol or dialkyl glycerol, as described in United

States Patent Application Serial No. 08/739,109, filed October 25, 1996, entitled "Vascular Endothelial Growth Factor (VEGF) Nucleic Acid Ligand Complexes." VEGF Nucleic Acid Ligands that are associated with a High Molecular Weight, Non-Immunogenic Compound, such as Polyethyleneglycol, or a Lipophilic Compound, such as Glycerolipid, phospholipid, or glycerol amide lipid, in a diagnostic or therapeutic complex are described in United States Patent Application Serial No. 08/897,351, filed July 21, 1997, entitled "Vascular Endothelial Growth Factor (VEGF) Nucleic Acid Complexes." Each of the above described patent applications which describe modifications of the basic SELEX procedure are specifically incorporated by reference herein in their entirety.

SELEX identifies Nucleic Acid Ligands that are able to bind targets with high affinity and with outstanding specificity, which represents a singular achievement that is unprecedented in the field of Nucleic Acids research. These characteristics are, of course, the desired properties one skilled in the art would seek in a therapeutic or diagnostic ligand.

In order to produce Nucleic Acid Ligands desirable for use as a pharmaceutical, it is preferred that the Nucleic Acid Ligand (1) binds to the target in a manner capable of achieving the desired effect on the target; (2) be as small as possible to obtain the desired effect; (3) be as stable as possible; and (4) be a specific ligand to the chosen target. In most situations, it is preferred that the Nucleic Acid Ligand has the highest possible affinity to the target. Additionally, Nucleic Acid Ligands can have facilitating properties.

In commonly assigned U.S. Patent Application Serial No. 07/964,624, filed October 21, 1992 ('624), now United States Patent No. 5,496,938, methods are described for obtaining improved Nucleic Acid Ligands after SELEX has been performed. The '624 application, entitled Methods of Producing Nucleic Acid Ligands, is specifically incorporated herein by reference.

The SELEX process has been used to identify a group of high affinity RNA Ligands to VEGF from random 2'-aminopyrimidine RNA libraries and ssDNA ligands from random ssDNA libraries (United States Patent Application Serial No. 08/447,169, filed May 19, 1995, entitled High-Affinity Oligonucleotide Ligands to Vascular Endothelial Growth Factor (VEGF), which is a Continuation-in-Part Application of United States Patent Application Serial No. 08/233,012, filed April 25, 1994, entitled High-Affinity

Oligonucleotide Ligands to Vascular Endothelial Growth Factor (VEGF), both of which are incorporated herein by reference; see also Green et al. (1995) Chemistry and Biology 2:683-695).

In embodiments where the VEGF Nucleic Acid Ligand(s) can serve in a targeting capacity, the VEGF Nucleic Acid Ligands adopt a three dimensional structure that must be retained in order for the VEGF Nucleic Acid Ligand to be able to bind its target. In embodiments where the Lipid Construct comprises a Complex and the VEGF Nucleic Acid Ligand of the Complex is projecting from the surface of the Lipid Construct, the VEGF Nucleic Acid Ligand must be properly oriented with respect to the surface of the Lipid Construct so that its target binding capacity is not compromised. This can be accomplished by attaching the VEGF Nucleic Acid Ligand at a position that is distant from the binding portion of the VEGF Nucleic Acid Ligand. The three dimensional structure and proper orientation can also be preserved by use of a Linker or Spacer as described supra.

Any variety of therapeutic or diagnostic agents can be attached to the Complex for targeted delivery by the Complex. In addition, any variety of therapeutic or diagnostic agents can be attached encapsulated, or incorporated into the Lipid Construct as discussed supra for targeted delivery by the Lipid Construct.

In embodiments where the Complex is comprised of a Lipophilic Compound and a VEGF Nucleic Acid Ligand in association with a Liposome, for example, the VEGF Nucleic Acid Ligand could target tumor cells expressing VEGF (e.g., in Kaposi's sarcoma) for delivery of an antitumor drug (e.g., daunorubicin) or imaging agent (e.g., radiolabels). It should be noted that cells and tissues surrounding the tumor may also express VEGF, and targeted delivery of an antitumor drug to these cells would also be effective.

In an alternative embodiment, the therapeutic or diagnostic agent to be delivered to the Target cell could be another Nucleic Acid Ligand.

It is further contemplated by this invention that the agent to be delivered can be incorporated into the Complex in such a way as to be associated with the outside surface of the Liposome (e.g., a prodrug, receptor antagonist, or radioactive substance for treatment or imaging). As with the VEGF Nucleic Acid Ligand, the agent can be

associated through covalent or Non-Covalent Interactions. The Liposome would provide targeted delivery of the agent extracellularly, with the Liposome serving as a Linker.

In another embodiment, a Non-Immunogenic, High Molecular Weight Compound (e.g., PEG) can be attached to the Liposome to provide Improved Pharmacokinetic Properties for the Complex. VEGF Nucleic Acid Ligands may be attached to the Liposome membrane or may be attached to a Non-Immunogenic, High Molecular Weight Compound which in turn is attached to the membrane. In this way, the Complex may be shielded from blood proteins and thus be made to circulate for extended periods of time while the VEGF Nucleic Acid Ligand is still sufficiently exposed to make contact with and bind to its Target.

In another embodiment of the present invention, more than one VEGF Nucleic Acid Ligand is attached to the surface of the same Liposome. This provides the possibility of bringing the same VEGF molecules in close proximity to each other and can be used to generate specific interactions between the VEGF molecules.

In an alternative embodiment of the present invention, VEGF Nucleic Acid Ligands and a Nucleic Acid Ligand to a different Target can be attached to the surface of the same Liposome. This provides the possibility of bringing VEGF in close proximity to a different Target and can be used to generate specific interactions between VEGF and the other Target. In addition to using the Liposome as a way of bringing Targets in close proximity, agents could be encapsulated in the Liposome to increase the intensity of the interaction.

The Lipid Construct comprising a Complex allows for the possibility of multiple binding interactions to VEGF. This, of course, depends on the number of VEGF Nucleic Acid Ligands per Complex, and the number of Complexes per Lipid Construct, and mobility of the VEGF Nucleic Acid Ligands and receptors in their respective membranes. Since the effective binding constant may increase as the product of the binding constant for each site, there is a substantial advantage to having multiple binding interactions. In other words, by having many VEGF Nucleic Acid Ligands attached to the Lipid Construct, and therefore creating multivalency, the effective affinity (i.e., the



avidity) of the multimeric Complex for its Target may become as good as the product of the binding constant for each site.

In certain embodiments of the invention, the Complex of the present invention is comprised of a VEGF Nucleic Acid Ligand attached to a Lipophilic Compound such as a glycerol lipid. In this case, the pharmacokinetic properties of the Complex will be improved relative to the VEGF Nucleic Acid Ligand alone. As discussed *supra*, the glycerol lipid, phospholipid or glycerol amide lipid may be covalently bound to the VEGF Nucleic Acid Ligand at numerous positions on the VEGF Nucleic Acid Ligand. In embodiments where a glycerol lipid is used, it is preferred that the VEGF Nucleic Acid Ligand is bonded to the lipid through phosphodiester linkages.

In another embodiment of the invention, the Lipid Construct comprises a VEGF Nucleic Acid Ligand or Complex. In this embodiment, the glycerolipid can assist in the incorporation of the VEGF Nucleic Acid Ligand into the Liposome due to the propensity for a glycerolipid to associate with other Lipophilic Compounds. The glycerolipid in association with a VEGF Nucleic Acid Ligand can be incorporated into the lipid bilayer of the Liposome by inclusion in the formulation or by loading into preformed Liposomes. The glycerolipid can associate with the membrane of the Liposome in such a way so as the VEGF Nucleic Acid Ligand is projecting into or out of the Liposome. In embodiments where the VEGF Nucleic Acid Ligand is projecting out of the Complex, the VEGF Nucleic Acid Ligand can serve in a targeting capacity. It is to be understood that additional compounds can be associated with the Lipid Construct to further improve the Pharmacokinetic Properties of the Lipid Construct. For example, a PEG may be attached to the exterior-facing part of the membrane of the Lipid Construct.

In other embodiments, the Complex of the present invention is comprised of a VEGF Nucleic Acid Ligand covalently linked to a Non-Immunogenic, High Molecular Weight Compound such as Polyalkylene Glycol or PEG. In this embodiment, the pharmacokinetic properties of the Complex are improved relative to the VEGF Nucleic Acid Ligand alone. The Polyalkylene Glycol or PEG may be covalently bound to a variety of positions on the VEGF Nucleic Acid Ligand. In embodiments where

Polyalkylene Glycol or PEG are used, it is preferred that the VEGF Nucleic Acid Ligand is bonded through the 5' hydroxyl group via a phosphodiester linkage.

In certain embodiments, a plurality of Nucleic Acid Ligands can be associated with a single Non-Immunogenic, High Molecular Weight Compound, such as Polyalkylene Glycol or PEG, or a Lipophilic Compound, such as a glycerolipid. The Nucleic Acid Ligands can all be to VEGF or VEGF and a different Target. In embodiments where there are multiple VEGF Nucleic Acid Ligands, there is an increase in avidity due to multiple binding interactions with VEGF. In yet further embodiments, a plurality of Polyalkylene Glycol, PEG, glycerol lipid molecules can be attached to each other. In these embodiments, one or more VEGF Nucleic Acid Ligands or Nucleic Acid Ligands to VEGF and other Targets can be associated with each Polyalkylene Glycol, PEG, or glycerol lipid. This also results in an increase in avidity of each Nucleic Acid Ligand to its Target. In embodiments where multiple VEGF Nucleic Acid Ligands are attached to Polyalkylene Glycol, PEG, or glycerol lipid, there is the possibility of bringing VEGF molecules in close proximity to each other in order to generate specific interactions between VEGF. Where multiple Nucleic Acid Ligands specific for VEGF and different Targets are attached to Polyalkylene Glycol, PEG, or glycerol lipid, there is the possibility of bringing VEGF and another Target in close proximity to each other in order to generate specific interactions between the VEGF and the other Target. In addition, in embodiments where there are Nucleic Acid Ligands to VEGF or Nucleic Acid Ligands to VEGF and different Targets associated with Polyalkylene Glycol, PEG, or glycerol lipid, a drug can also be associated with Polyalkylene Glycol, PEG, or glycerol lipid. Thus the Complex would provide targeted delivery of the drug, with Polyalkylene Glycol, PEG, or glycerol lipid serving as a Linker.

VEGF Nucleic Acid Ligands selectively bind VEGF. Thus, a Complex comprising a VEGF Nucleic Acid Ligand and a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound or a Lipid Construct comprising a VEGF Nucleic Acid Ligand or a Complex are useful as pharmaceuticals or diagnostic agents. The present invention, therefore, includes methods of inhibiting angiogenesis by administration of a Complex comprising VEGF Nucleic Acid Ligand and a Non-Immunogenic, High

Molecular Weight Compound or Lipophilic Compound, a Lipid Construct comprising VEGF Nucleic Acid Ligand or a Complex comprising a VEGF Nucleic Acid Ligand and a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound. The VEGF Nucleic Acid Ligand-containing Complexes and Lipid Constructs can be used to

5 treat, inhibit, prevent or diagnose any disease state that involves inappropriate VEGF production, particularly angiogenesis. Angiogenesis rarely occurs in healthy adults, except during the menstrual cycle and wound healing. Angiogenesis is a central feature, however, of various disease states, including, but not limited to cancer, diabetic retinopathy, macular degeneration, psoriasis and rheumatoid arthritis. The present invention, thus, also includes,

10 but is not limited to, methods of treating, inhibiting, preventing or diagnosing diabetic retinopathy, macular degeneration, psoriasis and rheumatoid arthritis. Additionally, VEGF is produced and secreted in varying amounts by virtually all tumor cells. Thus, the present invention, includes methods of treating, inhibiting, preventing, or diagnosing cancer by administration of a Complex comprising a VEGF Nucleic Acid Ligand and a Non-

15 Immunogenic, High Molecular Weight Compound or Lipophilic Compound, a Lipid Construct comprising a Complex, or a VEGF Nucleic Acid Ligand in association with a Lipid Construct without being part of the Complex. It has been shown that in a type of cancer, Kaposi's sarcoma (KS), cells not only produce abundant amounts of VEGF but also express functional VEGF receptors and therefore use VEGF for autocrine growth.

20 Thus, the present invention includes method of inhibiting Kaposi's Sarcoma by administration of a Complex comprising VEGF Nucleic Acid Ligand and a Non-Immunogenic, High Molecular Weight Compound or a Lipophilic Compound, a Lipid Construct comprising a Complex, or a VEGF Nucleic Acid Ligand in association with a Lipid Construct without being part of a Complex.

25 In one embodiment of the present invention, the Lipid Construct comprises a Complex comprised of a VEGF Nucleic Acid Ligand and a Lipophilic Compound with an additional diagnostic or therapeutic agent encapsulated in the Lipid Construct or associated with the interior of the Lipid Construct. In the preferred embodiment, the Lipid Construct is a Lipid Bilayer Vesicle, and more preferably a Liposome. The therapeutic use of

30 Liposomes includes the delivery of drugs which are normally toxic in the free form. In the

liposomal form, the toxic drug is occluded, and may be directed away from the tissues sensitive to the drug and targeted to selected areas. Liposomes can also be used therapeutically to release drugs over a prolonged period of time, reducing the frequency of administration. In addition, liposomes can provide a method for forming aqueous  
5 dispersions of hydrophobic or amphiphilic drugs, which are normally unsuitable for intravenous delivery.

In order for many drugs and imaging agents to have therapeutic or diagnostic potential, it is necessary for them to be delivered to the proper location in the body, and the liposome can thus be readily injected and form the basis for sustained release and drug  
10 delivery to specific cell types, or parts of the body. Several techniques can be employed to use liposomes to target encapsulated drugs to selected host tissues, and away from sensitive tissues. These techniques include manipulating the size of the liposomes, their net surface charge, and their route of administration. MLVs, primarily because they are relatively large, are usually rapidly taken up by the reticuloendothelial system (principally the liver  
15 and spleen). UVs, on the other hand, have been found to exhibit increased circulation times, decreased clearance rates and greater biodistribution relative to MLVs.

Passive delivery of liposomes involves the use of various routes of administration, e.g., intravenous, subcutaneous, intramuscular and topical. Each route produces differences in localization of the liposomes. Two common methods used to direct liposomes actively to  
20 selected target areas involve attachment of either antibodies or specific receptor ligands to the surface of the liposomes. In one embodiment of the present invention, the VEGF Nucleic Acid Ligand is associated with the outside surface of the liposome, and serves in a targeting capacity. Additional targeting components, such as antibodies or specific receptor ligands can be included on the liposome surface, as would be known to one of skill  
25 in the art. In addition, some efforts have been successful in targeting liposomes to tumors without the use of antibodies, see, for example, U.S. Patent No. 5,019,369, U.S. Patent No. 5,435,989, and U.S. Patent No. 4,441,775, and it would be known to one of skill in the art to incorporate these alternative targeting methods.

Therapeutic or diagnostic compositions of a Complex comprising VEGF Nucleic  
30 Acid Ligand and a Non-Immunogenic, High Molecular Weight Compound or Lipophilic

Compound, a Lipid Construct comprising a Complex comprised of a VEGF Nucleic Acid Ligand and a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound, and a VEGF Nucleic Acid Ligand in association with a Lipid Construct without being part of a Complex may be administered parenterally by injection, although  
5 other effective administration forms, such as intraarticular injection, inhalant mists, orally active formulations, transdermal iontophoresis or suppositories, are also envisioned. One preferred carrier is physiological saline solution, but it is contemplated that other pharmaceutically acceptable carriers may also be used. In one embodiment, it is envisioned that the carrier and the VEGF Nucleic Acid Ligand Complex constitute a physiologically-  
10 compatible, slow release formulation. The primary solvent in such a carrier may be either aqueous or non-aqueous in nature. In addition, the carrier may contain other pharmacologically-acceptable excipients for modifying or maintaining the pH, osmolality, viscosity, clarity, color, sterility, stability, rate of dissolution, or odor of the formulation. Similarly, the carrier may contain still other pharmacologically-acceptable excipients for  
15 modifying or maintaining the stability, rate of dissolution, release, or absorption of the VEGF Nucleic Acid Ligand. Such excipients are those substances usually and customarily employed to formulate dosages for parental administration in either unit dose or multi-dose form.

Once the therapeutic or diagnostic composition has been formulated, it may be  
20 stored in sterile vials as a solution, suspension, gel, emulsion, solid, or dehydrated or lyophilized powder. Such formulations may be stored either in ready to use form or requiring reconstitution immediately prior to administration. The manner of administering formulations containing VEGF Nucleic Acid Ligand for systemic delivery may be via subcutaneous, intramuscular, intravenous, intranasal or vaginal or rectal suppository.

25 The advantages of the Complexes and Lipid Constructs of the invention include: i) improving the plasma pharmacokinetics of the Nucleic Acid Ligand; ii) presenting Nucleic Acid Ligands in a multivalent array with the aim of increasing the avidity of interaction with their targets; iii) combining two or more presenting Nucleic Acid Ligands with different specificities in the same liposome particle; iv) enhancing the delivery of presenting  
30 Nucleic Acid Ligands to tumors by taking advantage of the intrinsic tumor targeting

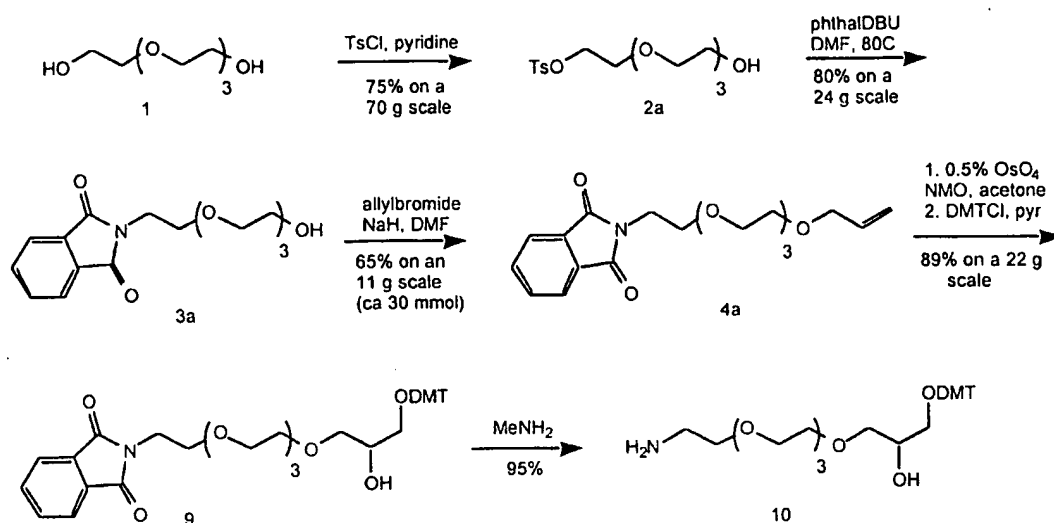
properties of liposomes; and v) using the high affinity and specificity of presenting Nucleic Acid Ligands, which is comparable to that of antibodies, to guide liposomal contents to specific targets. presenting Nucleic Acid Ligands are well suited for the kinds of preparations described here since, unlike most proteins, the denaturation of presenting  
5 Nucleic Acid Ligands by heat, various molecular denaturants and organic solvents is readily reversible.

The following examples are provided to explain and illustrate the present invention and are not to be taken as limiting of the invention. The structures of the Nucleic Acid Ligands described in the examples below are shown in **Figure 1**. Example 1 describes the  
10 conjugation of Nucleic Acid Ligands with lipid reagents. The ability of a dialkylglycerol derivative of the VEGF Nucleic Acid Ligand (NX278), either as a free ligand or incorporated in the bilayer of liposomes (NX278-L), to inhibit the activity of VEGF in vitro and in vivo is described in Example 2. Example 3 describes the experimental procedures for generating 2'-F pyrimidine modified RNA ligands to VEGF. Example 4 describes the  
15 2'-F pyrimidine -modified RNA ligands to VEGF. Example 5 describes the synthesis of glycerolipid, phospholipid, and glycerol amide lipid, and PEG-modified VEGF Nucleic Acid Ligands. Example 6 describes the pharmacokinetic properties of phospholipid (PL) and PEG modified VEGF Nucleic Acid Ligands. Example 7 describes preparations of NX31838 PL -Liposome Complex, Examples 8-10 describe the in vivo efficacy of VEGF  
20 Nucleic Acid Ligand Complexes. Example 11 describes the intravitreal pharmacokinetics of NX31838-40K PEG in rabbits.

**EXAMPLE 1. Synthesis of a dialkyl glycerol (1,2-di-O-octadecyl-sn-glycerol)--modified VEGF Nucleic Acid Ligand.**

25 In this example, conjugation of Nucleic Acid Ligands with lipid reagents is described. Synthesis of (1,2-di-O-octadecyl-sn-glycerol)--modified VEGF Nucleic Acid Ligand is shown below.

Scheme 1



5

**Tetraethylene glycol monotosylate (2a):** Tetraethylene glycol (200 mL, 1.15 mol) was dissolved in 500 mL of pyridine and cooled to  $0^\circ\text{C}$  and treated with 22.0 g (0.115 mol) of p-toluenesulfonylchloride. When solution was complete, the reaction mixture was stored in the refrigerator overnight, and then concentrated *in vacuo*. The residue was dissolved in 800 mL of EtOAc and extracted with 3 x 600 mL of  $\text{H}_2\text{O}$ . The  $\text{H}_2\text{O}$  fractions were back-extracted with EtOAc, and the combined EtOAc fractions were extracted with saturated aqueous  $\text{Na}_2\text{HPO}_4$ . The organic phase was dried over  $\text{MgSO}_4$  and concentrated to a colorless oil. The oil was purified by flash chromatography using 800 mL of silica gel and eluting with hexane, 25% EtOAc-50% EtOAc in hexane, then EtOAc, then 10% MeOH-20% MeOH in EtOAc to afford 23.7 g (60%) of pure product and 11% of product containing a minor impurity. **2a:**  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  7.77 (d,  $J=8.1$  Hz, 2H), 7.32 (d,  $J=8.1$  Hz, 2H), 4.13 (t,  $J=4.8$  Hz, 2H), 3.68-3.53 (m, 14H), 2.58 (t,  $J=5.6$  Hz, 1H), 2.42 (s, 3H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  168.2, 158.3, 144.8, 135.9, 133.8, 132.0, 129.9, 128.0, 127.7, 126.6, 123.1, 113.0, 85.9, 73.0, 70.6, 70.4, 70.0, 69.7, 67.8, 64.4, 55.1, 37.1; Low resolution MS  $m/e$  calculated for  $\text{C}_{15}\text{H}_{24}\text{O}_8\text{S}$  ( $M+1$ ): 349.1.

20

**Tetraethylene glycol monophthalimide (3a):** To a stirred solution of 31.96 g (0.092 mol) of **2a** in 400 mL of anhydrous DMF was added 14.2 g (1.05 equiv.) of

phthalimide and 14.4 mL (1.05 equiv.) of 1,8-diazabicyclo[5.4.0]undec-7-ene. The solution was heated at 70° C for 18 h then concentrated *in vacuo*. The crude yellow oil was purified by flash chromatography using 1600 mL of silica gel and eluting with 25% EtOAc-50% EtOAc-75% EtOAc in hexane, then EtOAc, then 10% MeOH-20% MeOH in EtOAc to afford 23.8 g (80%) of **3a** as an oil. Upon standing, **3a** became a waxy white solid. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 7.84-7.78 (m, 2H), 7.70-7.66 (m, 2H), 3.86 (t, J=5.6 Hz, 2H), 3.70 (t, J=5.6 Hz, 2H), 3.64-3.51 (m, 12H), 2.67 (bs, 1H); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ 168.2, 133.8, 132.0, 123.1, 72.4, 70.5, 70.4, 70.2, 70.0, 67.8, 61.6, 37.2.

**Synthesis of compound 4a:** A solution of 15 g (0.0464 mol) of **3a** in 150 mL of THF and 15 mL of DMF was cooled to 0° C under Ar. Allyl bromide (6.0 mL, 1.5 equiv.) was added to the solution, followed by addition of 1.76 g (1.5 equiv.) of NaH as a solid. The opaque yellow suspension was stirred at 0° C for 30 minutes and then at room temperature for 18 hr. MeOH (50-100 mL) was added and concentrated then mixture was concentrated *in vacuo*. The crude material was purified by flash chromatography using 1500 mL of silica gel and eluting with 25% EtOAc-50% EtOAc-75% EtOAc in hexane, then EtOAc, then 10% MeOH in EtOAc to afford 11.05 g (65%) of **4a** as a yellow oil. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 7.84-7.80 (m, 2H), 7.72-7.67 (m, 2H), 5.94-5.84 (m, 1H), 5.28-5.14 (m, 2H), 3.99 (d, J=5.61 Hz, 2H), 3.88 (t, J=5.85 Hz, 2H), 3.72 (t, J=5.76 Hz, 2H), 3.64-3.54 (m, 13H); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ 168.0, 134.6, 133.7, 131.9, 123.0, 116.9, 72.0, 70.4, 69.9, 69.2, 67.7, 37.0.

**1-Dimethoxytrityl-3-(phthalimidotetraethyleneglycolyl)-sn-glycerol (9):**

According to **Scheme 1**, compound **9** was synthesized as follows: To a stirred solution of **4a** (10.13 g, 0.0279 mol) in 100 mL of acetone and 1 mL of H<sub>2</sub>O was added 3.98 g (1.22 equiv.) of N-methylmorpholine N-oxide. To this suspension was added 1.75 mL (0.005 equiv.) of Osmium tetroxide as a 2.5% solution in iPrOH. After addition of the OsO<sub>4</sub> solution, the reaction mixture became clear yellow. After TLC analysis indicated complete conversion of **4a** (ca 16 h), the reaction mixture was treated with 1.5 g of sodium hydrosulfite and 5.0 g of florisil and stirred 30 minutes. The suspension was filtered through florisil, the filtrate was concentrated to an oil. This crude product was combined with another batch prepared in the same manner from 1.0 g of **4a**. Two 100 mL portions of

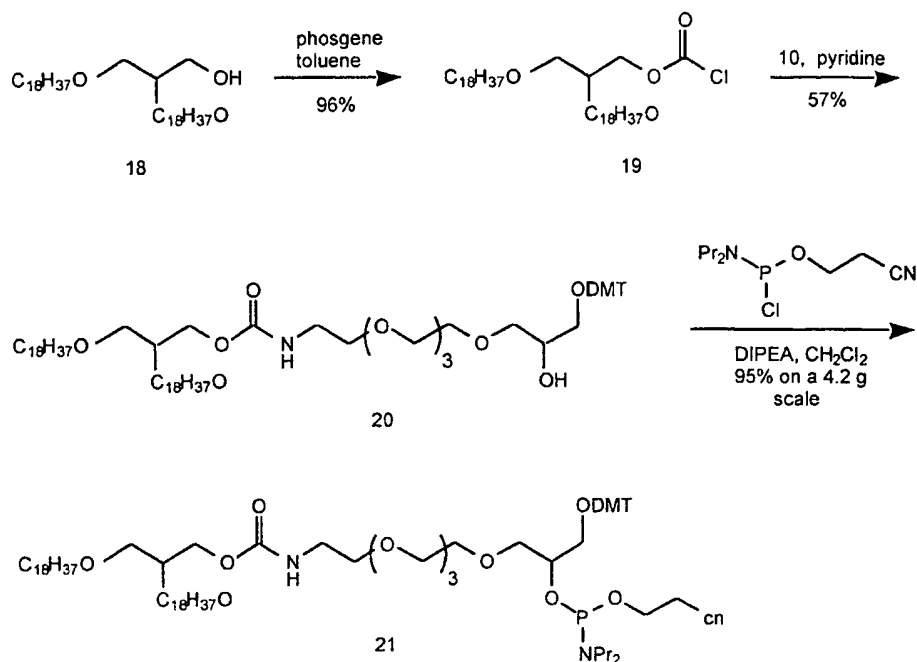


pyridine were co-evaporated from the combined lots and the residue was dissolved in 300 mL pyridine. The solution was cooled to 0° C and 10.89 g (1.05 equiv.) of 4,4'-dimethoxytritylchloride was added. A drying tube was inserted in the flask and the reaction mixture was stirred at room temperature 16 h. The solution was treated with 20 mL of MeOH and concentrated *in vacuo*, keeping the temperature of the water bath below 40° C. The crude oil was purified by flash chromatography using 1100 mL of silica gel (wet-packed onto column using 3% triethylamine in hexane) and eluting with 10-100% EtOAc in hexane (all containing 3% triethylamine) to give 21.3 g (89% after two steps) of **9** as a yellow oil. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 7.80-7.77 (m, 2H), 7.66-7.64 (m, 2H), 7.39-7.22 (m, 9H), 7.20-6.76 (m, 4H), 3.97 (bs, 1H), 3.84 (t, J=5.97 Hz, 2H), 3.74 (s, 6H), 3.68 (t, J=5.7 Hz, 2H), 3.60-3.49 (m, 14H), 3.13-2.76 (m, 2H), 2.00 (bs, 1H); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ 168.2, 158.3, 144.8, 135.9, 133.8, 132.0, 129.9, 128.0, 127.7, 126.6, 123.1, 113.0, 85.9, 73.0, 70.6, 70.4, 70.0, 69.7, 67.8, 64.4, 55.1, 37.1; Low resolution MS m/e calculated for C<sub>40</sub>H<sub>45</sub>O<sub>10</sub>N (M+NH<sub>4</sub><sup>+</sup>): 717.5.

**15            1-Dimethoxytrityl-3-(aminotetraethylenglycolyl)-sn-glycerol (10):**

According to **Scheme 1**, compound **10** was synthesized as follows: Compound **9** (5.2 g, 7.2 mmol) was taken up in 50 mL of 40% methylamine in H<sub>2</sub>O and 10 mL of methanol was added to solublize the starting material. The reaction mixture was heated at 50°C for 5 hr, and then was concentrated *in vacuo* and coevaporated with toluene. The crude material was purified by flash chromatography on 200 mL of silica gel, eluting with 15% methanolic ammonia in dichloromethane. Collected 3.94g (96%) of **10** as a pale yellow oil. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 7.46-7.21 (m, 9H, DMT), 6.81 (d, 4H, DMT), 4.00 (m, 1H), 3.80 (s, 6H), 3.70-3.49 (overlapping m, 18H), 3.20 (dd, J=9.24, 5.49 Hz, 1H), 3.12 (dd, J=9.21, 6.0 Hz, 1H), 2.84-2.80 (m, 3H); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ 158.30, 144.82, 136.01, 129.95, 128.04, 127.66, 126.61, 112.95, 85.85, 73.46, 72.85, 70.55, 70.45, 69.99, 69.51, 64.43, 55.10, 41.40; Low resolution MS m/e calculated for C<sub>32</sub>H<sub>44</sub>O<sub>8</sub>N (M+1<sup>+</sup>): 570.353, found 570.4.

## Scheme 2



5

**Chloroformate 19:** To a stirred solution of 3 g (5.03 mmol) of 1,2-di-*O*-octadecyl-*sn*-glycerol **18** in 60 mL of toluene was added 20 mL of a 1.93 M solution of phosgene. Additional phosgene solution (2 X 10 mL; 15.4 equiv phosgene total) was added until no further alcohol starting material remained (by <sup>1</sup>H NMR analysis of concentrated aliquots).

- 10 The excess phosgene and HCl was removed by aspirator and the reaction mixture was concentrated *in vacuo* to afford 3.3 g (98%) of the desired chloroformate **19** as a white powder. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 4.45 (dd, J=11.22, 3.69 Hz, 1H), 4.34 (dd, J=11.22, 6.15 Hz, 1H), 3.65 (m, 1H), 3.56-3.40 (m, 6H), 1.53 (m, 4H), 1.24 (m, 62H), 0.87 (t, J=6.36 Hz, 6H); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ 75.90, 71.91, 71.35, 70.93, 69.36, 31.99, 29.96-  
15 29.44 (overlapping signals from hydrocarbon chains), 26.13, 26.04, 22.76, 14.18.

- Conjugate 20:** To a stirred solution of 2.25 g (3.95 mmol) of **10** in 60 mL of pyridine was added 2.6 g of the distearyl glycerol chloroformate **18**. <sup>1</sup>H NMR analysis of a concentrated aliquot after 2 h revealed no remaining chloroformate and the mixture was concentrated *in vacuo*. The crude residue was combined with material similarly prepared  
20 from 0.5 g (0.88 mmol) of **10** and 0.58 g of the chloroformate and the combined lots

purified by flash silica gel chromatography on a column of 100 mL of silica gel (packed in hexanes containing 2% triethylamine) eluting with 200 mL hexanes, then 250 mL each of 10-20 and 30% EtOAc in hexanes, 500 mL 40% EtOAc in hexanes, then 250 mL each of 50-60-70 and 80% EtOAc in hexanes, and finally with 250 mL of EtOAc. The product  
5 containing fractions were concentrated to afford 3.3 g (57%) of the conjugate **20**.

**Phosphoramidite 21:** To a stirred solution of 3.8 g (3.26 mmol) of the conjugate in 25 mL of  $\text{CH}_2\text{Cl}_2$  was added 1.14 mL (6.52 mmol) of diisopropylethylamine then 1.09 mL (4.88 mmol) of 2-cyanoethyl N,N-diisopropylchloro-phosphoramidite. After 2 hours, the mixture was diluted with  $\text{CH}_2\text{Cl}_2$  and washed with saturated  $\text{NaHCO}_3$  solution, dried over  
10  $\text{Na}_2\text{SO}_4$ , and concentrated. The crude residue was purified by flash silica gel chromatography on a column of 125 mL of silica gel (packed in hexanes containing 2% triethylamine) eluting with 100 mL hexanes, then 250 mL each of 10 and 20% EtOAc in hexanes, 500 mL 30% EtOAc in hexanes, then 250 mL of 50% EtOAc in hexanes. The product containing fractions were concentrated to afford 4.2 g (95%) of the  
15 phosphoramidite **21**.  $^{31}\text{P}$  NMR ( $\text{CDCl}_3$ )  $\delta$  151.52, 151.08.

**The VEGF Nucleic Acid Ligand - 1,2-di-O-octadecyl-sn-glycerol conjugate**

The 1,2-di-O-octadecyl-sn-glycerol group was conjugated to VEGF Nucleic Acid Ligand NX213 (See **Figure 1A**) using phosphoramidite 21 (Scheme 2). The resulting conjugate was named NX278 (See **Figure 1B**). NX278 was purified by reverse phase  
20 HPLC and its composition was confirmed by electrospray mass spectroscopy ( $m/z$  observed = 11703.4,  $m/z$  calculated = 11720). Phosphorothioate internucleoside linkages were used at 8 positions in NX278 (at the 3' and 5' ends) and the difference of .16 mass units between the expected and observed masses is probably due to incomplete oxidation by the sulfurizing agent resulting, on average, in one less phosphorothioate linkage per molecule  
25 than expected.

**EXAMPLE 2. In vitro and in vivo efficacy of Nucleic Acid Ligand-Liposome Complex. Dialkylglycerol (DAG)-modified VEGF Nucleic Acid Ligand (NX278) embedded in Liposome bilayer.**

NX278-Liposome Complex was prepared by incubating NX-278 (1 mg) (**Figure 1B**; SEQ ID NO: 2 ) with a spray-dried mixture of DSPC:cholesterol (50 mg/ml; 2:1, Mol:Mol) in 25 mM phosphate (pH 7.4) buffer containing 9% sucrose and sonicated for 15-30 min at approximately 60 degrees C using a probe-type sonicator until opalescent solution was obtained. The control Nucleic Acid Ligand-Liposome Complex containing a sequence scrambled analog of ligand NX-278 (scNX278) (**Figure 1C**; SEQ ID NO:3 ) was prepared in the same manner. In a typical preparation, liposomes with a mean diameter of 50 nm and a distribution width at half height of 20 nm were obtained. The size of Liposome particles was determined in a particle analyzer (Leeds & Northrup Model Microtrack UPA 150, Horsham, PA). Liposomes of comparable size distribution were obtained with the same lipid composition but without the lipid-conjugated Nucleic Acid Ligand. A 50 nm liposome is expected to contain an average of 40 Nucleic Acid Ligands, displayed on both sides of the bilayer. The calculation was made as follows. Assuming a surface area of 19 C for cholesterol and 60 C for distearylphosphatidylcholine in the liposome, a number of lipid molecules per liposome of  $3.13 \times 10^4$  was obtained, for a spherical liposome with 50 nm outer diameter and membrane thickness of 20 C. From the composition of the liposome (2:1 mol:mol distearylphosphatidylcholine(MW=790.2):cholesterol(MW=386.7)), assuming homogeneous distribution of lipids, molecular mass of  $2.1 \times 10^7$  for the liposome was calculated.

To determine the partitioning of the Nucleic Acid Ligands between the inside and outside surfaces of liposomes, the accessibility of NX278 in the liposomal formulation to  $T_1$  ribonuclease was examined. With two riboguanosines in the sequence (Green et al. (1995) Chemistry and Biology 2:683-695), NX278 is efficiently cleaved by ribonuclease  $T_1$ . Simple incubation of NX278 with preformed liposomes does not protect the Nucleic Acid Ligand from ribonuclease  $T_1$ . However, when NX278 is incorporated in liposomes by sonication (NX278-Liposome), about 1/3 is protected from the nuclease. The addition of 0.1% triton X-100 to NX278-Liposome, which disrupts the liposomes without affecting the activity of the nuclease, exposes the previously protected Nucleic Acid Ligand to digestion. These results are consistent with the notion that the Nucleic Acid Ligand is distributed on both sides of the bilayer.

### **Binding affinities of NX213, NX278, and NX278-Liposome for VEGF**

The binding affinities of NX213, NX278 and NX278-Liposome for VEGF were examined using a competition electrophoretic mobility shift method (Figure 2). The binding affinity of NX278 for VEGF was comparable to that of NX213. The apparent binding affinity of NX278-Liposome was 3-fold lower compared with NX278. A part of the observed affinity reduction is potentially due to the confinement of a fraction of the Nucleic Acid Ligand to the liposome interior. As expected, the sequence scrambled analogs bind to VEGF with substantially lower affinities (Figure 2).

### **Plasma pharmacokinetic properties of NX213, NX278, and NX278-Liposome**

The concentrations of NX213, NX278 and NX278-Liposome in the plasma of Sprague Dawley rats as a function of time are shown in Figure 15, and the parameters from compartmental analysis are summarized in Table 1. The majority of NX213 is cleared rapidly in the alpha phase with a  $t_{1/2}$  of 7 minutes and an overall clearance rate of 6.8 ml/kg/min. Conjugation of a phospholipid group to the Nucleic Acid Ligand results in highly biphasic clearance from the blood with increased  $\beta(t_{1/2})$  and somewhat slower overall rate of clearance (4.95 ml/kg/min) relative to NX213. Incorporation of NX278 into a liposome shows a substantial additional decrease in clearance of the Nucleic Acid Ligand from plasma (1.88 ml/kg/min).

### **The Effect of NX278 on HUVEC proliferation and angiogenesis**

The effects of NX278-liposome, scNX278-liposome and NX213 on the proliferation of human umbilical vein endothelial cells (HUVEC) was examined. HUVECs were grown in the presence of VEGF (10 ng/ml) in IMDM:Ham's F12 (1:1) medium containing 10% fetal calf serum (FCS) and heparin (45 :g/ml). Cells were plated in 24-well gelatin-coated plates at a density of 20,000 cells per well on day zero and treated with the above ligands at concentrations between 0.1 nM to 1 :M on days 1, 2, and 3 (replacing the media along with the ligands. NX278-Liposome inhibited the proliferation of HUVECs with an IC<sub>50</sub> of  $\approx 300$  nM (the concentration refers to the Nucleic Acid Ligand

component); scNX278-Liposome and NX213 were significantly less effective ( $IC_{50} > 1 \mu M$ ).

VEGF induces angiogenesis in chicken allantoic membrane (CAM) assays, and this assay can be utilized to study compounds that inhibit angiogenesis. The assay is done by placing filter discs soaked in VEGF on the CAM and the development of new blood vessels can be quantitated. NX278-Liposome effectively blocked VEGF induced angiogenesis (data not shown), while NX213, NX278, and scNX278-Liposome had no effect. Together these studies demonstrate that NX278 is a specific inhibitor of VEGF induced endothelial cell proliferation in vitro and new vessel formation in vivo.

#### **Effect of NX278 on VEGF induced capillary permeability**

VEGF is the only known angiogenic factor that transiently enhances capillary permeability. The ability of NX278-Liposome to inhibit the vascular permeability activity of VEGF in vivo was examined. The vascular permeability assay (also known as the Miles assay (Miles, A. A. and Miles, E. M. (1952) *J. Physiol.* (London) **118**:228) was performed in guinea pigs essentially as described (Senger, R. S. *et al.*, (1983) *Science* **219**:983). NX278-Liposome, NX278, and NX213 at the concentration of  $1 \mu M$  were injected intradermally with VEGF (20 nM) in guinea pigs preinjected with Evans blue dye. In response to VEGF, an increase in vascular permeability causes extravasation of albumin-bound Evans blue dye resulting in a blue spot at the site of injection. Because the recovery of the dye by organic solvent extraction is generally very poor, a quantitation method has been developed that measures the absorption of light through the skin. NX213, NX278, NX278-Liposome and neutralizing monoclonal antibody to VEGF all significantly inhibited VEGF-induced permeability as shown in **Figure 3**. Among the Nucleic Acid Ligands, NX278-Liposome appeared to be the most potent antagonist. Sequence scrambled analogs of these compounds were not inhibitory. The differences were dramatic and noticeable to the naked eye.

**NX278-L inhibits Kaposi's sarcoma cell lines in vitro**

Inhibitors of VEGF have a potential utility in a variety of diseases, including malignancies where tumor progression and metastasis are dependent on new vessel formation. While most tumor types are known to produce VEGF, previously none has been shown to express functional VEGF receptors. It has been shown recently that Kaposi's Sarcoma (KS) cells not only produce abundant amounts of VEGF but also express functional VEGF receptors and therefore use VEGF for autocrine growth. KS cell lines thus provide a unique opportunity to examine the ability of NX278 to interrupt the autocrine VEGF growth activity.

The effects of NX278-Liposome, scNX278-Liposome and NX213 on the proliferation of KS cells was examined. KS cell line KSY-1 was plated in 24-well gelatin coated plates at a density of 7,500-10,000 cells per well on day zero in medium containing RPMI 1640 supplemented with 2% FCS, L-glutamine, penicillin and streptomycin. Nucleic Acid Ligands were added at concentrations between 0.1 nM to 1  $\mu$ M in fresh medium on day 1, 2, and 3 and the cell count was performed on day 4. NX278-Liposome inhibited the proliferation of KS cells with an IC<sub>50</sub> of 100 nM; at 1  $\mu$ M NX278-Liposome, the growth of these cells was completely inhibited. scNX278-Liposome and NX213 exhibited IC<sub>50</sub> values of >1  $\mu$ M (**Figure 4**).

**NX278-Liposome inhibits KS cell growth in vivo.**

Because VEGF is a growth factor for KS cells, the effect of VEGF antagonists on KS tumors in vivo is likely to be two-fold: inhibition of paracrine growth effect of VEGF on tumor associated endothelial cells and inhibition of autocrine growth effect on tumor cells. KS tumors may thus be particularly sensitive to VEGF antagonists. To test the activity of the Nucleic Acid Ligands in vivo, tumor trocars (3 mm<sup>3</sup>) were implanted in athymic mice on day one and treated for five consecutive days beginning on day two with 50, 100 or 150  $\mu$ g/day/mouse. The rate of tumor growth was measured for a period of two weeks. NX278-Liposome inhibited the tumor growth in a dose dependent manner with very little inhibition of tumor growth at the lowest dose level of 50  $\mu$ g/day/mouse dose (**Figure 5A**), and marked inhibition of tumor growth at both 100 and 150  $\mu$ g/day/mouse dose levels

(Figure 5B, 150 µg/day/mouse shown). Empty liposomes (Figure 5A, B), scNX278-Liposome as well as NX213 and NX278 were ineffective at all doses examined. In addition, NX278-Liposome blocked the VEGF-induced fluid leakage from blood vessels.

5   **Example 3. Experimental Procedures for 2'-Fluoro Pyrimidine - Modified RNA Ligands to VEGF**

This example provides general procedures followed and incorporated in Example 4 for the evolution of 2'-Fluoro-modified Nucleic Acid Ligands to VEGF.

**Materials**

10       Recombinant human VEGF<sub>165</sub> purified from the insect cell-line Sf 21 was purchased from R & D Systems as a carrier-free lyophilized powder. The protein was resuspended in phosphate-buffered saline to a concentration of 10 µM and stored at -20°C in small aliquots until use. Aliquots were stored at 4° C for up to 4 weeks after thawing. Sf 21-expressed mouse VEGF<sub>164</sub>, and *E. coli*-expressed human VEGF<sub>121</sub>, VEGF/PIGF  
15 heterodimer, and PIGF were also purchased from R & D Systems as carrier-free, lyophilized preparations.

Oligonucleotides were purchased from Operon Technologies, Inc. or were synthesized using an Applied Biosystems Model 394 oligonucleotide synthesizer according to optimized protocols. 2'-F-and 2'-OMe-ribonucleotide phosphoramidites  
20 were prepared by JBL Scientific, Inc. (San Luis Obispo, CA). 2'-F-pyrimidine NTPs were also purchased from JBL. 2'-OH-purine NTPs and dNTPs were from Pharmacia Biotech, Piscataway, NJ.

*T. aquaticus* thermostable DNA polymerase (Taq polymerase) was purchased from Perkin Elmer-Cetus, (Foster City, CA); AMV reverse transcriptase (AMV RT) was  
25 from Life Sciences, Inc.; Klenow DNA polymerase was from New England Biolabs, Beverly, MA. T7 RNA polymerase was from Enzyco, Inc. (Denver, CO). Sequenase DNA polymerase is produced by United States Biochemical Corp. (Cleveland, OH).

α-[<sup>32</sup>P]-ATP and γ-[<sup>32</sup>P]-ATP were obtained from New England Nuclear (Boston, MA).



### The SELEX protocol

The SELEX procedure has been described in detail in the SELEX Patent Applications. Chemically synthesized DNA oligonucleotide libraries ("30N7" and "40N7") were prepared with randomized regions of 30 or 40 nucleotides flanked by common 5' and 3' fixed sequences (5'-*TAATACGACTCACTATAGGGAGGACGATGCGG*(30 or 40 N)*CAGACGACTCGCCCGA-3'*; SEQ ID NO:). Italicized nucleotides at the 5' end of each template correspond to the T7 RNA polymerase promoter sequence. Oligonucleotide primers were also synthesized for use in template preparation and amplification, and reverse transcription: 5'-TCGGGCGAGTCGTCTG-3' ("3N7"; SEQ ID NO:) and 5'-TAATACGACTCACTATAGGGAGGACGATGCGG-3' ("5N7" SEQ ID NO:). Double-stranded DNA templates were prepared by annealing primer 3N7 to the 30N7 or 40N7 libraries and extending the primer using Klenow DNA polymerase or AMV RT. The higher temperature of incubation used for AMV RT (45°C rather than 37°C) may better promote complete extension through highly structured template oligonucleotides. The libraries were transcribed using T7 RNA polymerase in the presence of 1 mM each 2'-OH-ATP and GTP, 3 mM each 2'-F-CTP and UTP, and 50  $\mu$ Ci  $\alpha$ -<sup>32</sup>P-ATP. RNAs were purified from denaturing polyacrylamide gels by excising the gel slice containing the RNA, crushing it, and soaking for an extended time in 2mM EDTA.

The SELEX process of affinity selection followed by selected pool amplification has been described in detail (See the SELEX Patent Applications). In brief, one round of selection and amplification was performed as follows: VEGF was mixed with a 5- or 10-fold excess of RNA in phosphate-buffered saline with 1 mM MgCl<sub>2</sub> (PBSM) (30N7 and 40N7 libraries) or in Tris-buffered saline, 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub> (TBSMC) (30N7 library only), and the mixture was serially diluted by three. After incubation at 37°C for 15 minutes, the mixtures were passed through 0.45  $\mu$  Type HA filters (Millipore) to collect complexes of VEGF with RNA. RNAs were eluted from selected filters by incubation in 2:1 phenol, pH 7:7 M urea. After precipitation from the aqueous phase,

RNAs were annealed to primer 3N7 and reverse transcribed using AMV RT. The resultant cDNAs were amplified with 15 cycles of the polymerase chain reaction (PCR) using the 3N7 and 5N7 primers and Taq DNA polymerase. Transcription of the PCR product yielded a new library enriched for sequences with affinity for VEGF. At round 4, a substantial background filter-binding signal in the absence of VEGF had emerged in all three selected RNA pools. To deplete the pools of filter-binding RNAs, rounds 5 and 6 were performed with an alternative scheme for partitioning VEGF-bound RNAs from unbound molecules: after incubation of the RNA pool with the growth factor, each mixture was applied to an 8% polyacrylamide, non-denaturing gel and electrophoresed at 10 W for 45-60 minutes at 4°C. VEGF/RNA complexes migrated above the unbound RNA in this system and were visualized by exposure of X-ray film to the gel. For these rounds, selected RNAs were purified by the crush and soak method, as described above. After twelve rounds of selection and amplification, individual molecules in the selected pools were cloned using the pCR-Script Direct Cloning kit from Stratagene (La Jolla, CA). Plasmids were purified using the alkaline lysis method (PERFECTprep Plasmid DNA kit, 5 Prime → 3 Prime, Boulder, CO) and sequences of the cloned regions were obtained using the Dye Terminator Cycle Sequencing kit available from Perkin Elmer (Foster City, CA). Fluorescent sequencing ladders were read at the National Jewish Center, laboratory of Brian Kotzin, Denver, CO. Sequences were grouped into families and aligned by eye.

### Measurement of binding affinities

Nucleic Acid Ligands radiolabeled during transcription by incorporation of  $\alpha$ - $[^{32}\text{P}]$ -labeled NTPs, or after synthesis using  $\gamma$ - $[^{32}\text{P}]$ -ATP and T4 polynucleotide kinase, were incubated in low concentration (between 20 and 70pM) with varying concentrations of VEGF or other growth factors at 37°C for 15 minutes. Incubations were in TBS, PBS, or HEPES-buffered saline (HBS), pH 7.4, with or without the addition of supplemental divalent cations. Samples were passed through prewashed 0.45  $\mu$  Type HA filters (Millipore) followed by a 5-10 ml wash with binding buffer. Filters were immersed in scintillant and counted to quantitate the amount of protein-bound RNA retained by each

filter. The equilibrium dissociation constant ( $K_D$ ) of Nucleic Acid Ligand binding to a specific protein was calculated from the data points as described in Green et al. (1996) Biochem. 35: 14413-14424.

#### 5 Affinity selection of Nucleic Acid Ligand fragments

Ten pmol internally-radiolabeled transcripts of high affinity VEGF Nucleic Acid Ligands were partially digested with S7 nuclease to generate a mixture of radiolabeled fragments. One-tenth of the fragmented RNA was incubated with 10 pM VEGF in 45 ml binding buffer, prior to filtration through nitrocellulose. Selected fragments recovered from the filter were run out on a high resolution denaturing polyacrylamide gel next to a lane loaded with the unselected fragment pool. The smallest selected bands were individually purified from the gel and further labeled at their 5' ends with polynucleotide kinase to increase their specific activity. One-half of the sample was annealed to a cDNA of the original transcript and extended to the end of the template using Sequenase DNA polymerase. Comparison of the migration of the purified fragment and its extension product to a standard sequencing ladder was used to determine the probable size and position of the selected fragment within the original transcript. Synthetic oligonucleotides corresponding in sequence to the affinity selected fragments were prepared to verify that the truncated Nucleic Acid Ligand retained affinity for VEGF.

20

#### 2'-OMe-substitution

The 2'-OMe substitution experiments were performed essentially as described in Green et al. (1995) Chem. Biol. 2:683-695. Three or four libraries were prepared for each of three truncated ligands (t22, t2, t44) in which five or six 2'-OH-purine positions were partially 2'-OMe-substituted. Each purine position was partially 2'-OMe-modified in only one of the libraries. Each 5'-radiolabeled library was incubated with VEGF, and substituted oligonucleotides bound by the protein were collected on nitrocellulose filters. The selected pool and the starting unselected library were partially hydrolyzed by alkali and the products were displayed on a high resolution polyacrylamide gel. A "band intensity ratio" was determined for each purine position by dividing the phosphorimage

30

signal obtained from hydrolysis at that position in the selected pool by the signal obtained for the same position in the unselected library. Band intensity ratios that fall well above the range for a particular position are indicative of a bias for 2'-OH (against 2'-OMe) in the affinity selected pool.

5

### Binding rate constants

A small amount (typically less than 1 pmol) of 5'-radiolabeled Nucleic Acid Ligands were incubated with 1 nM VEGF at 37°C in 1 ml buffered saline supplemented with divalent cations. At time "zero," 50 µl were filtered through nitrocellulose to determine the fraction of RNA bound to protein, then an excess (100 or 500 nM in different experiments) of unlabeled Nucleic Acid Ligand was added and 50 µl aliquots were filtered at time points thereafter. Filters were counted in scintillant to determine the amount of radiolabeled RNA still bound to VEGF at each time point. The data, plotted as fraction of RNA bound ( $f$ ) vs time, was fitted to an equation for exponential decay:

$$f(t) = f_0 e^{-kt} + b,$$

where  $f_0$  is the fraction of RNA bound at time zero,  $k$  is the dissociation rate constant ( $k_d$ ) and  $b$  is the residual binding of radiolabeled RNA to the filter at the end of the experiment (effectively, in the absence of protein). Association rate constants ( $k_a$ s) were calculated from the measured  $k_d$  and  $K_D$  values according to the equation:

$$k_a = k_d / K_D$$

20

### Example 4. 2'-Fluoro-Modified RNA Ligands to VEGF

#### Selection of Ligands

Ligands to VEGF were isolated in three separate SELEX experiments from libraries of 2'-F-pyrimidine-modified RNAs containing 30 or 40 random nucleotides. Selections were performed in PBS supplemented with 1 mM  $MgCl_2$  (30N and 40N libraries) or in Tris-buffered saline with 1 mM  $MgCl_2$  and 1 mM  $CaCl_2$  (30N library only). Approximately  $10^{14}$  unique sequences were included in the first selection cycle of each experiment. After ten cycles, the affinity between VEGF and each RNA pool had

improved approximately 1000-fold relative to the starting pools. As no further improvement in binding affinity was observed after two additional cycles, individual members of the twelfth round pools were cloned and sequences were determined for about 50 isolates from each selection.

5           Oligonucleotide ligands to VEGF<sub>165</sub> were isolated in three separate SELEX experiments. Individual clones were isolated and sequenced and the sequences grouped into families based on shared primary structural motifs (**Table 2**). The name of each ligand indicates the target (V=VEGF), the selection buffer (P=PBS; T=TBS), the length of the randomized region in the library (30 or 40 nucleotides) and the clone number  
10 (following the decimal). The frequency with which a sequence appeared among the clones analyzed is indicated in parentheses; sequences that differed by only one nucleotide were attributed to PCR mutagenesis of a common precursor and were grouped together with the variable base indicated in the sequence by the appropriate symbol (Y=U or C). The fixed sequences common to all ligands are shown in lower case letters at the  
15 top. For individual clones the sequence of the variable region is shown in upper case. For some ligands, fixed region sequences in lower case are appended to the variable region sequence where they contribute to possible secondary structures. The high affinity K<sub>d</sub> for binding to VEGF is shown for each ligand. One ligand in each family was selected for further analysis (gray box).

20           Of a total of 143 clones analyzed, 76 sequences differing by more than one nucleotide were obtained. 44 of these sequences could be grouped into three major families based on conserved primary structural motifs (**Table 2**). Sequences that may be grouped in minor families with five or fewer members and "orphan" sequences that were unique among the isolates are shown in **Table 6**. Ligands containing the primary  
25 structural motif defined by Families 1 and 2 arose in all three affinity selections. Similarities between the conserved primary structures of both families suggest that they may also share similar secondary structures and/or that they may interface with VEGF using similar contact regions. Members of Family 2 share the possibility of forming a short basepaired stem enclosing the conserved sequence motif in a large "loop"  
30 (underlined in **Table 2**). With the exception of the closing A/U basepair, the sequence

identity of bases in the putative stem regions is not conserved. Such "co-variation" of bases that conserves secondary rather than primary structure supports the existence of the putative stem and suggests that this structure may be important for the high affinity conformation of this family of VEGF ligands. No similarly conserved basepairing interactions were detected among Family 1 sequences. A third family of ligands arose only in the selections performed in TBSMC (Family 3, **Table 2**). In addition to a highly conserved primary structure motif, in all members of this family, sequences 3' of the conserved region share basepairing complementarity to nucleotides in the 5' fixed region (underlined in **Table 2**). Since, for most of the ligands, the bases on the 5' side of the putative stem cannot be said to covary with their basepairing partners, this observation is less predictive of a common secondary structure; nevertheless, our initial guess for a minimal high affinity sequence derived from this family (described below) was guided by the strong conservation of this motif. The affinities of the individual RNA ligands for VEGF were estimated based on a single determination of the  $K_D$  for their interaction. With few exceptions, the ligands showed very high affinity for the growth factor, with  $K_D$ s between 5 and 50 pM.

### Minimal Ligands

The shared primary and secondary structural motifs that define each sequence family hint at the minimal sequence elements required for high affinity binding to VEGF. Nested truncations of a representative ligand from each family (indicated by gray boxes in **Table 2**) were produced by chemical synthesis and their relative affinities for VEGF were determined (**Table 3**). Truncated versions of ligands VP30.22, VP30.2 and VT30.44 were prepared by chemical synthesis and their affinities for VEGF were determined as described in Example 3. Initial truncations (t22, t2, t44) were further refined by synthesis of oligonucleotides with additional bases lacking from the 5' and/or 3' ends. In order to initiate the chemical synthesis, the 3'-most nucleotide of several of the ligands was modified either by substitution of 2'-OH-cytidine for 2'-F-cytidine

(underlined) or by addition of a 3'-3'-linked deoxythymidine "cap" (asterisks). The length of each oligonucleotide (minus the cap) and its high affinity  $K_D$  for binding to VEGF are shown.

An initial prediction for the minimal sequence from clone VP30.22 (Family 1) was made by mapping the ends of a purified, affinity-selected fragment of the full-length ligand (see Example 3). This 29 nucleotide molecule ("t22") showed an approximately three-fold loss in binding affinity for VEGF relative to the full length ligand. Further truncation at the 3' end of this molecule caused a precipitous loss in affinity but up to 6 additional nucleotides could be removed from the 5' end with little or no consequence (Table 3). For clone VP30.2 from Family 2 and clone VT30.44 from Family 3, truncated ligands "t2" and "t44" were synthesized that encompassed the putative five basepair stem and all of the conserved sequence motif. Both truncated ligands retained nearly all of the binding activity of the full length molecule. Further truncation by deleting one putative basepair at a time (one nucleotide from each end of the ligand) caused a gradual loss in affinity. Thus, for these sequences, truncations based on possible secondary structures predicted very well the minimal high affinity ligand, and further supports the hypothesis that the putative stems contribute to the high affinity conformation of these ligands.

### 2'-OMe modification

Substitution at the 2'-OH positions of RNA oligonucleotides by 2'OMe has been observed to improve their stability against nucleases present in rat urine as well as in other biological fluids. Stabilization of oligonucleotides to nucleases is likely to be critical to their success as therapeutic or diagnostic agents. Unfortunately, 2'-OMe-modified nucleoside triphosphates are not generally accepted as substrates by RNA polymerases under standard reaction conditions. However, 2'-OMe purines may be introduced into a specific oligonucleotide by chemical synthesis. It has been observed that some high affinity 2'-OH purine RNA ligands will accept a surprisingly high

percentage of 2'-OMe purine substitutions with little loss of affinity for the target protein. To identify those purine positions for which 2'-OMe substitution is compatible with high affinity binding to VEGF, several syntheses of ligands t2, t22 and t44 were prepared in which five or six purines at a time were partially substituted with the modified nucleotide (described in Example 3). Affinity selection of each partially substituted library was used to isolate those molecules that retained substantial affinity for VEGF. In such an affinity selected pool, positions that do not tolerate substitution are biased for 2'-OH and thus show higher sensitivity to hydrolysis by alkali relative to the same position in the unselected library. 5'-radiolabeled unselected and affinity selected pools were partially hydrolysed by alkali and the products were displayed on a high resolution polyacrylamide gel. In ligand t22, G10 and A12 showed substantial bias for 2'OH in the affinity selected pool, as did A6 and G21 in ligand t2, and A5 and A6 in ligand t44. While the foregoing analysis identifies those positions that are likely to disallow substitution with 2'OMe nucleotides, one cannot predict from these data how simultaneous modification of all other purines will affect binding affinity. In fact, ligand t22, synthesized with all 2'-OMe-purines except G10, A12 and G22 (which showed a marginal preference for 2'-OH), bound to VEGF with an affinity equal to if not better than the all 2'-OH-purine sequence (Table 4).

Truncated oligonucleotides (t22, t2, and t44) were chemically synthesized with all but one, two or three purine positions substituted with 2'-OMe-purines. The remaining 2'-OH-purines are indicated in each ligand name and are shown in bold in the ligand sequence. K<sub>D</sub>s for the binding of each substituted ligand to VEGF are shown.

Further substitution at G22 had little effect on binding to VEGF, but incorporation of 2'-OMe at G10 or A12, as predicted, was detrimental to binding affinity. Similarly, ligands t2 and t44 tolerated 2'-OMe-substitution at all but two purines with a three- to four-fold impact on the affinity of the Nucleic Acid Ligand for VEGF (Table 4).



### Binding affinities and rate constants for substituted truncates

In the hope of identifying highly 2'-substituted VEGF Nucleic Acid Ligands of minimal length, all 2'-OMe-substitutions that did not dramatically decrease binding were incorporated into truncated ligands t22c, t2a, and t44a (see Table 3). 2'OH nucleotides are indicated in bold, and 2'OMe nucleotides are indicated in plain text. The resultant Nucleic Acid Ligands, t22-OMe and t44-OMe, bound to VEGF with  $K_D$ s of 67 pM and 49 pM, respectively, while ligand t2OMe bound with a  $K_D$  of approximately 140 pM (Table 5). These  $K_D$ s compare favorably with that of NX-213 ( $K_D$ =140 pM), a 2'-NH<sub>2</sub>-pyrimidine-, 2'-OMe-purine-substituted oligonucleotide inhibitor of VEGF described previously (see United States Patent Application No. 08/447,169, which is incorporated herein by reference). Each of the truncated 2'-OMe-substituted oligonucleotides was found to compete with NX-213 and with one another for binding to VEGF.

Dissociation rate constants ( $k_d$ ) were determined for each of the three 2'-OMe-substituted ligands by following the loss of a preformed complex between radiolabeled ligand and VEGF upon the addition of a large excess of unlabeled ligand. Ligand t22-OMe showed the fastest rate of dissociation with a half life of approximately 60 seconds. Ligands t2-OMe and t44-OMe showed slightly slower rates of dissociation with half lives on the order of 170 and 90 seconds, respectively. Association rate constants ( $k_a$ ), calculated from the equilibrium dissociation constant and the dissociation rate constant ( $K_D=k_d/k_a$ ), ranged from  $3 \times 10^7$  to  $2 \times 10^8 \text{ M}^{-1}\text{sec}^{-1}$  (Table 5). Such rapid rates of association suggest a near diffusion limited binding interaction between these ligands and VEGF, and are in line with the association rate constants observed for SELEX-derived Nucleic Acid Ligands to other targets.

### Divalent cation dependence

Ligands in Families 1 and 2 were selected in the presence of magnesium cations while Family 3 ligands were selected in a buffer containing both magnesium and calcium.

Since divalent cations may contribute to RNA/protein interactions through nonspecific or specific stabilization of high affinity RNA structures, we asked whether magnesium and/or calcium were required for the high affinity binding of representative ligands to VEGF. The affinities of Nucleic Acid Ligands t22-OMe and t2-OMe (from Families 1  
5 and 2, respectively) were unchanged in the presence or absence of supplemental divalent cations or the chelating agent EDTA (data not shown). However, Family 3 ligands, as represented by ligand t44-OMe, showed an absolute dependence on the presence of calcium for high affinity binding to VEGF. Binding was dramatically reduced ( $K_D > 10^{-7}$ ) when divalent cations in the binding buffer were replaced with EGTA. The addition of  
10 excess  $MgCl_2$  to divalent-cation-depleted binding buffer gave no improvement in binding affinity, but  $CaCl_2$ , in two-fold molar excess over EGTA, fully restored binding activity. Identical binding behavior was observed for the unmodified ligand t44 (data not shown).

### Protein specificity

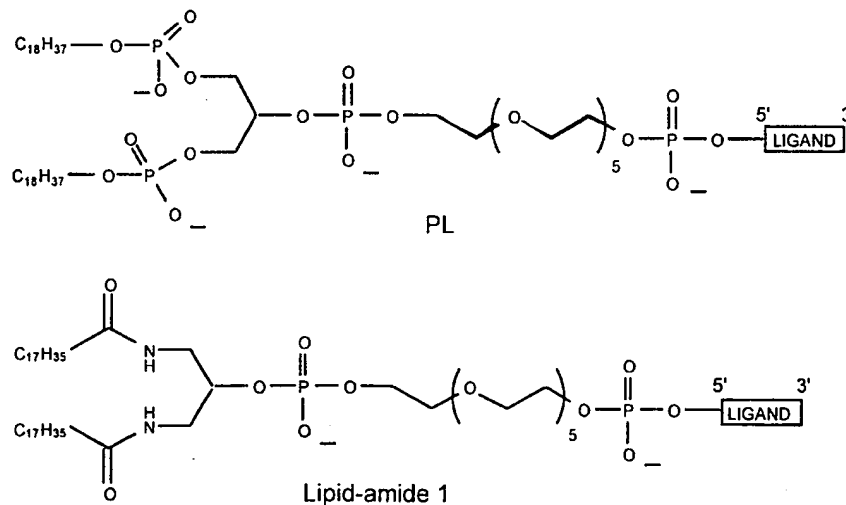
15 The oligonucleotides described here were selected based on their affinities for VEGF<sub>165</sub>, the larger of two diffusible isoforms of the growth factor. VEGF<sub>121</sub>, the smaller isoform, lacks one of the exons in VEGF<sub>165</sub> and, unlike the latter, does not bind to heparin. None of the three truncated, 2'-OMe-substituted oligonucleotides bound with any measurable affinity to VEGF<sub>121</sub>. Furthermore, the native structure of VEGF<sub>165</sub> is  
20 essential for the binding of all three Nucleic Acid Ligands, as no binding is observed when the protein is reduced with DTT prior to incubation with the oligonucleotides.

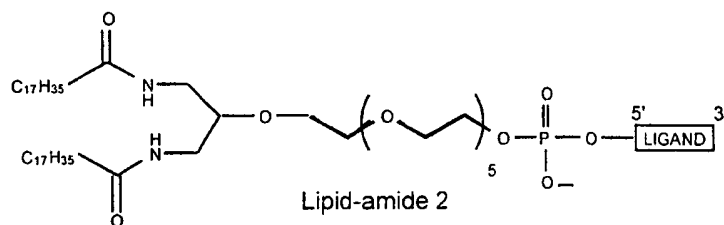
VEGF is a highly conserved protein across species, the human VEGF<sub>165</sub> and mouse VEGF<sub>164</sub> isoforms showing 88% sequence identity. The truncated, 2'-OMe-substituted ligands bound equally well to human and murine VEGF. However, no  
25 binding was observed for any of the ligands to homodimers of PlGF, a placenta-derived protein that shares 53% sequence identity with VEGF across the conserved platelet derived growth factor-like domain. Heterodimers between VEGF and PlGF have recently

been isolated from the supernatants of both normal and tumor-derived cell lines, and such heterodimers show activity in binding to one of two high affinity VEGF receptors and in inducing responses in cultured endothelial cells. The biological relevance of VEGF/PlGF heterodimers is unknown. Substantial binding, though with greatly reduced affinities, was observed with VEGF/PlGF heterodimers. These data may indicate that the Nucleic Acid Ligands bind at or near the interface between the two subunits in a dimer and that PlGF does not present all of the contact sites necessary for high affinity binding. Alternatively, the structure of the VEGF subunit may be altered by participation in a heterodimer with PlGF with consequent distortion of the Nucleic Acid Ligand binding surface.

**Example 5. Synthesis of phospholipid, glycerol amide lipid, and PEG - modified VEGF Nucleic Acid Ligands.**

Three different formulations were used for the synthesis of various Lipophilic Compound/Nucleic Acid Ligand Complexes as follows:

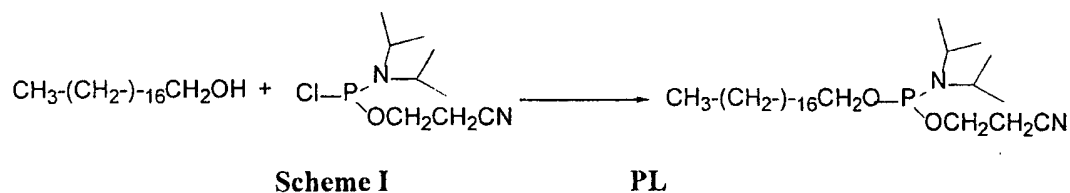




### 1. C-18 Phosphoramidite for the synthesis of PL formulation

An outline for the preparation of C-18 phosphoramidite is shown in Scheme I. 1-

- 5 Octadecanol was phosphorylated under standard condition. After work up the reaction mixture, the residue was purified on silica gel column with hexane : ethyl acetate : triethylamine (90 : 10 : 5) to offer 21.5 g of pure product (57% yield).



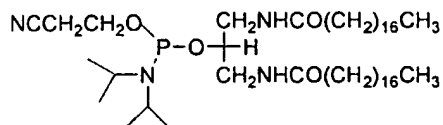
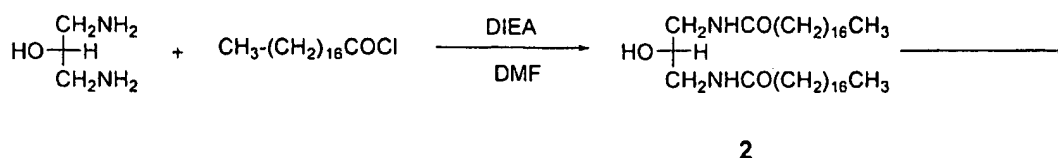
10

### II. Synthesis of Lipid Amide 1

This phosphoramidite, unlike the above PL, has amide linkages. The structure of the oligo resulting from conjugation of this lipid is shown below.

- Several experiment demonstrated that high insolubility of compound **2** in organic  
 15 solvents made NMR and MS characterization and further phosphitylation of compound **2** to DAG amidite **1** impossible, however, from the results for preparation of Lipid-spacer amidite (Scheme I), we expected the phosphitylation of compound **2** with chloro-(2-cyanoethoxy)-N,N-diisopropylamino-phosphine might go if refluxed the mixture. The approach to prepare the DAG amidite was shown in Scheme II.

20



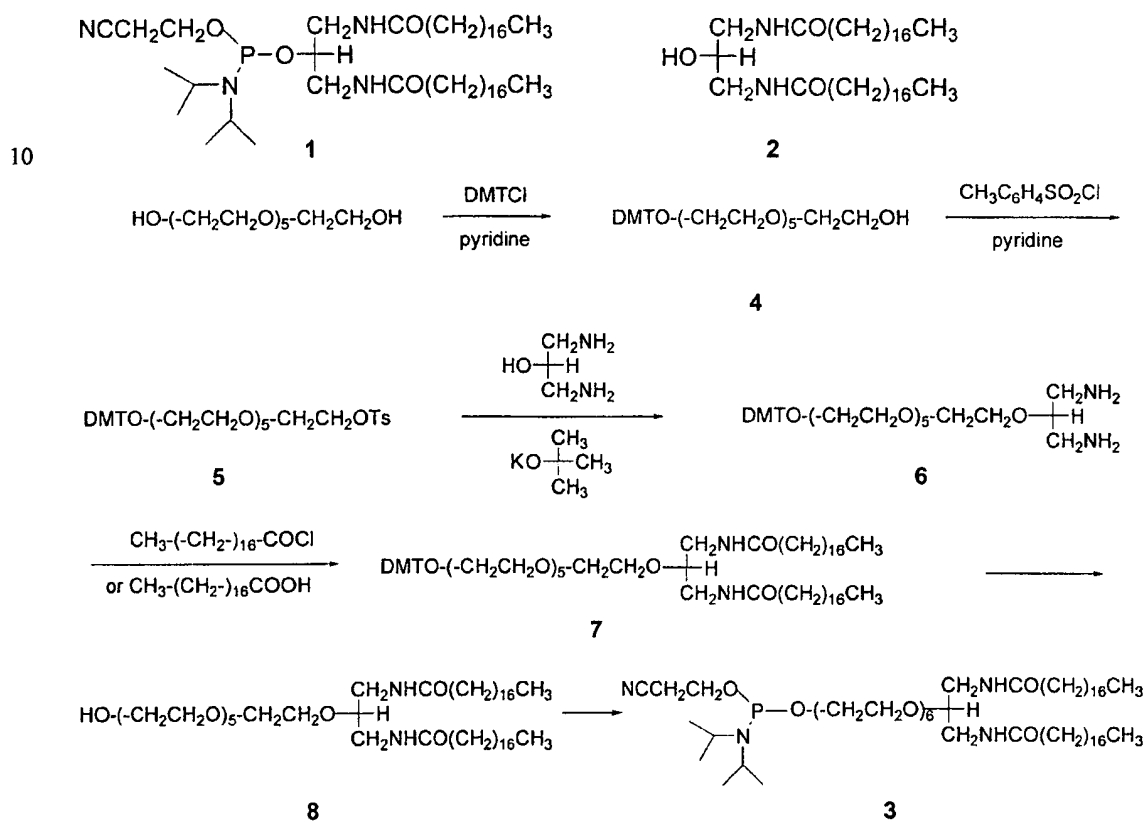
## Scheme II

**N,N'-Bis(stearoyl)-1,3-diaminopropanol-2 (2).** A solution of stearoyl chloride (6.789g, 22.41 mmol) in  $\text{ClCH}_2\text{CH}_2\text{Cl}$  (50 mL) was added dropwise to a solution of 1,3-diamino-2-hydropropane (1.0g, 11.10 mmol) in  $\text{ClCH}_2\text{CH}_2\text{Cl}$  (100.0 mL) and TEA (2.896g, 22.41 mmol) with stirring in R.T. After finishing addition, the mixture was heated to 70°C overnight, and clear solution was formed, and the solution was cooled to R.T., filtered, and the solids were washed with  $\text{CH}_2\text{Cl}_2$ ,  $\text{CH}_3\text{OH}$ , 5%  $\text{NaHCO}_3$  and ethyl ether, and dried in *vacuo* to give **2** (6.40g, 93% yield) as white solids.  $^1\text{H}$  NMR (pyridine- $d_5$ ; 60°C,  $\delta$ , ppm): 3.82-3.78 (m, 1H), 2.37 (t,  $J = 7.5$  Hz, 4H), 1.81-1.76 (m, 4H), 1.30-1.27 (m, 60H), 0.87 (t,  $J = 5.7$  Hz, 6H).

**N,N'-Bis(stearoyl)-O-(diisopropylamino-2-cyanoethoxyphosphinyl)-1,3-diaminopropanol-2 (1).** Compound **2** (5.80g, 9.31 mmol), dried overnight in *vacuo*, was in anhydrous  $\text{CH}_2\text{Cl}_2$  (150.0 mL) and N,N-diisopropylethylamine (4.2 mL, 18.62 mmol) was injected. The mixture was cooled in an ice-water bath and chloro-(2-cyanoethoxy)-N,N-diisopropylamino-phosphine (8.6 mL, 0.47 mmol) was injected. After stirring for 30 min, the mixture was heated at 60°C for 90 min. After cooling to R.T., insoluble materials were filtered and solution was washed with 5%  $\text{NaHCO}_3$  and brine, dried over  $\text{Na}_2\text{SO}_4$ , and concentrated in vacuum. The crude product was purified by precipitated from  $\text{CH}_3\text{CN}$  to afford pure product (4.65g, 61% yield) as white solids.  $^{31}\text{P}$  NMR ( $\text{CDCl}_3$ , ppm): 154.04.

### I. Synthesis of DAG-Spacer Amidite, Lipid Amide 2

Hexa(ethylene glycol) was incorporated in lipid amidite in order to alleviate the solubility of diamide compound **2**, which is a immediate intermediate to lipid amidite **1**. An outline of the preparation of lipid-spacer amidite **3** is shown in Scheme I. The coupling step of compound **5** with 1,3-diamino-2-hydroxypropane and potassium *t*-butoxide in THF did not go well, and the yield was only about 20%. One attempt to improve yield was made by reacting **5** and diamide **2**, however, no desired product was detected.



Scheme I

Lipid Amide 2

(4,4'-Dimethoxytrityloxy)-hexaethylene glycol (**4**). Hexa(ethylene glycol)(18.93g, 67.05 mmol) was covevaporated with anhydrous pyridine (3 x 50 mL), dissolved in anhydrous pyridine (400 mL), and, after cooling to 0°C, DMTrCl(23.85g, 70.40 mmol) in pyridine (50 mL) added dropwise during 30 min with stirring under Ar. The reaction

mixture was kept at R. T. overnight. The pyridine was removed under high vacuum and residue was dissolved in  $\text{CH}_2\text{Cl}_2$ , which was washed with 5%  $\text{NaHCO}_3$  and brine, dried over  $\text{Na}_2\text{SO}_4$ , and concentrated in vacuum. The crude product was purified by wet-flash silica gel column chromatography in a gradient of ethyl acetate, then  $\text{CH}_2\text{Cl}_2$  and  
5 methanol (95/5) containing 0.5% TEA. The appropriate fractions were combined, evaporated, and dried in vacuum to give **4** (26.1g, 66.6% yield) as a light yellow oil.  $^1\text{H}$  NMR ( $\text{DMSO}-d_6$ ;  $\delta$ , ppm): 7.40 (d,  $J = 7.2$  Hz, 2H), 7.33-7.24 (m, 7H), 6.89 (d,  $J = 8.9$  Hz, 4H), 4.61 (t,  $J = 5.1$  Hz, 1H), 3.73 (s, 6H), 3.05 (m, 24H);  $^{13}\text{C}$  NMR ( $\text{DMSO}-d_6$ ;  $\delta$ , ppm): 158.02, 145.02, 135.78, 129.67, 128.13, 127.71, 126.61, 113.14, 85.29, 72.33,  
10 72.27, 70.06, 69.87, 69.80, 69.75, 69.70, 62.84, 60.25, 60.19, 55.01.

**(4,4'-Dimethoxytrityloxy)-hexaethylene glycol tosylate (5).** To an ice cooled solution ( $0^\circ\text{C}$ ) of **4** in anhydrous pyridine (50 mL), was added a solution of toluene sulfonyl chloride in pyridine (30 mL). After 2 h at R. T., the solution was evaporated to a light yellow oil. The residue was taken-up in  $\text{CH}_2\text{Cl}_2$  and washed with 5%  $\text{NaHCO}_3$  and brine,  
15 dried over  $\text{Na}_2\text{SO}_4$ , filtered and evaporated in *vacuo*. The product was purified by wet-flash silica gel chromatography, eluting with ethyl acetate to give the product (4.08g, 93% yield) as light yellow oil.  $^1\text{H}$  NMR ( $\text{DMSO}-d_6$ ;  $\delta$ , ppm): 7.78 (d,  $J = 8.3$  Hz, 2H), 7.46 (d,  $J = 8.1$  Hz, 2H), 7.40 (d,  $J = 7.4$  Hz, 2H), 7.32-7.23 (m, 7H), 6.88 (d,  $J = 8.8$  Hz, 4H), 4.09 (t,  $J = 4.3$  Hz, 2H), 3.72 (s, 6H), 3.06 (m, 22H), 2.40 (s, 3H);  $^{13}\text{C}$  NMR  
20 ( $\text{DMSO}-d_6$ ;  $\delta$ , ppm): 158.01, 145.01, 135.78, 132.38, 130.12, 129.67, 128.12, 128.02, 127.80, 127.70, 127.62, 113.13.

**2-(4,4'-Dimethoxytrityloxy)-hexaethylene glycol-1,3-diaminopropane (6).** A mixture of 1,3-diamino-2-hydroxypropane (747mg, 8.28 mmol) and potassium *t*-butoxide (2.78g, 24.84 mmol) in anhydrous THF was heated to  $70^\circ\text{C}$  for 2h and then cooled to R.T.  
25 Compound **5** (4.08g, 5.25 mmol) in THF was injected, and the mixture was stirred at  $70^\circ\text{C}$  overnight until TLC showed on more **5** was left. After the solution was cooled to R.T., THF was removed in *vacuo*, and 25 mL of  $\text{CH}_2\text{Cl}_2$  and 25 mL water were added. The  $\text{CH}_2\text{Cl}_2$  layer was separated, and the water later was extracted with  $\text{CH}_2\text{Cl}_2$ . The  $\text{CH}_2\text{Cl}_2$  solutions were combined, dried over  $\text{Na}_2\text{SO}_4$  and evaporated under reduced

pressure. The crude product (2.43g) was directly used for reaction without further purification. <sup>1</sup>H NMR (DMSO-d<sub>6</sub>; δ, ppm): 7.41 (d, J = 7.7 Hz, 2H), 7.32-7.21 (m, 7H), 6.87 (d, J = 8.8 Hz, 4H), 3.73 (s, 6H), 3.52-3.40 (m, 24H), 3.17 (s, 1H), 3.07-3.02 (m, 4H).

- 5 **N,N'-Bis(stearoyl)-2-(4,4'-dimethoxytrityloxy)-hexaethyleneglycol-1,3-diaminopropane (7).** A solution of stearoyl chloride (3.363g, 11.1 mmol) in ClCH<sub>2</sub>CH<sub>2</sub>Cl was injected to a solution of **6** in ClCH<sub>2</sub>CH<sub>2</sub>Cl and TEA (1.9 mL, 11.1 mmol) with stirring in R.T. The mixture was kept at R.T. for 2h, then heated to 70°C overnight. After the solution was cooled to R.T., the solution was washed with 5%  
 10 NaHCO<sub>3</sub> and brine, dried over Na<sub>2</sub>SO<sub>4</sub>, and concentrated in vacuum. The crude product was purified by wet-flash silica gel column chromatography in a gradient of ethyl acetate and CH<sub>2</sub>Cl<sub>2</sub> (50/50) and then ethyl acetate and methanol (50/50). The second fraction was collected, evaporated, and dried in vacuum to give **7** (640mg) as a light yellow solid. <sup>1</sup>H NMR (DMSO-d<sub>6</sub>; δ, ppm): 7.40 (d, J = 7.2 Hz, 2H), 7.37-7.20 (m, 7H), 6.74 (d, J =  
 15 8.9 Hz, 4H), 3.71 (s, 6H), 3.63-3.51 (m, 24H), 3.17 (s, 1H), 3.16-3.13 (m, 4H), 2.12 (t, J = 7.3 Hz, 4H), 1.18 (m, 60H), 0.80 (t, J = 6.2 Hz, 6H).

- N,N'-Bis(stearoyl)-2-hexaethylene glycol-1,3-diaminopropane (8).** A mixture of compound **7** (640mg), 2.5% DCA solution in CH<sub>2</sub>Cl<sub>2</sub> (5 mL) and trihexylsilane (2 mL) was stirred at R.T. until orange color turned to pale color. After removal of CH<sub>2</sub>Cl<sub>2</sub>, the  
 20 residue was repeatedly precipitated from hexane to give a light yellow solid (210mg, 63% yield). <sup>1</sup>H NMR (CDCl<sub>3</sub>, δ, ppm): 3.3.69-3.59 (m, 24H), 3.17 (s, 1H), 3.06-3.01 (m, 4H), 2.21 (t, J = 7.9 Hz, 4H), 1.18 (m, 60H), 0.81 (t, J = 6.3 Hz, 6H).

- N,N'-Bis(stearoyl)-2-(diisopropylamino-2-cyanoethoxyphosphinyl)-hexaethylene glycol-1,3-diaminopropane (3).** Compound **8** (210mg, 0.237 mmol), dried overnight in  
 25 *vacuo*, was dissolved in anhydrous CH<sub>2</sub>Cl<sub>2</sub> (5.0 mL) and N,N-diisopropylethylamine (218 μL, 1.25 mmol) was added. The solution was cooled in an ice-water bath and chloro-(2-cyanoethoxy)-N,N-diisopropylamino-phosphine (106 μL, 0.47 mmol) was injected. After stirring for 30 min, the reaction mixture was diluted with CH<sub>2</sub>Cl<sub>2</sub> and washed with



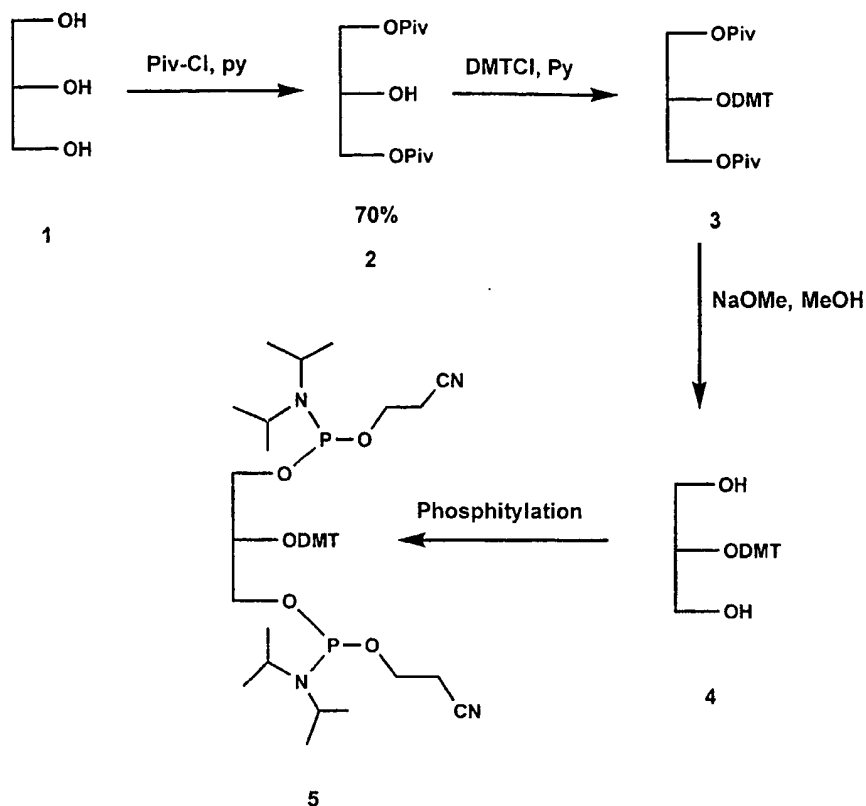
5% NaHCO<sub>3</sub> and brine, dried over Na<sub>2</sub>SO<sub>4</sub>, and concentrated in vacuum to afford <sup>31</sup>P NMR (CDCl<sub>3</sub>, ppm): 154.04.

#### Conjugation of 20K or 40K PEG NHS ester to VEGF Nucleic Acid Ligands

- 5 General procedure: VEGF oligonucleotide was exchanged for Triethylammonium salt and lyophilysed. The crude oligonucleotide was dissolved in 100mM sodium borate buffer (pH 9) to 60 mg /ml concentration. 2 Eq of PEG NHS ester (Shearwater Polymers, Inc.) was dissolved in dry DMF (Ratio of borate : DMF 1:1), and the mixture was warmed to dissolve the PEG NHS ester. The oligonucleotide solution was quickly added  
10 to PEG solution and the mixture was vigorously stirred at room temperature for 10 min. About 90% of the oligonucleotide becomes conjugated to PEG NHS ester. See **Figures 1H and 1I**.

#### Synthesis of Dimeric VEGF Nucleic Acid Ligands

- The dimeric VEGF Nucleic Acid Ligands shown in **Figures 1J, K, and L** were  
15 made as follows.



### Synthesis of 1,3-Dipivaloyl-2-O-dimethoxy tritylglycerol 3

To a stirred pyridine solution of compound 2 (62g of 70% pure product,  
 200mmol, in 200 ml of pyridine), prepared according to McGee *et al.* (1988, Synthetic  
 5 Communication, 1651), was added dimethoxy trityl chloride (84g, 240 mmol, 1.2-fold  
 excess) and the reaction was allowed to stir at room temperature for 16 hours. The  
 reaction mixture was concentrated under reduced pressure and the residue was taken in  
 CH<sub>2</sub>Cl<sub>2</sub>, (1L) washed with water, and dried (MgSO<sub>4</sub>) and concentrated. The crude mixture  
 10 (130g) was used as such in the next reaction.

### Synthesis of 2-O-dimethoxy trityl glycerol 4

A mixture of crude compound 2 (130g), NaOMe (28g) and methanol (900 ml)  
 was heated at 50°C for 16h. After the reaction was complete (TLC), the mixture was  
 15 concentrated to dryness and the residue was dissolved in water and CH<sub>2</sub>Cl<sub>2</sub> (1:1). Organic

layer was separated, and the aqueous layer was washed with saturated  $\text{NH}_4\text{Cl}$ , water and brine and dried ( $\text{MgSO}_4$ ). Evaporation of the solvent afforded a gummy compound, which was purified by silica gel column using 1:1 hexane/ethyl acetate containing 2% TEA to afford compound 4 in 75% isolated yield.  $^1\text{H}$  NMR ( $\text{DMSO}-d_6$ ) 3.02 - 3.07 (m, 2H), 3.17 - 3.23 (m, 2H), 3.3 - 3.35 (m, 1H), 3,7 (s, 6H), 4.26 (t,  $J = 4.1$  Hz, 2H,  $\text{D}_2\text{O}$  exchangeable), 6.59 - 6.86 (m, 4H), 7.17 - 7.68 (m, 9H).

### Synthesis of Bisamidite 5

To an ice cold stirred solution of the alcohol 4 (16.2g, 41.1868 mmol) in  $\text{CH}_2\text{Cl}_2$  (125 ml) and diisopropyl ethylamine (58 ml, 320 mmol) was added phosphitylating reagent (20.5 ml, 90.62 mmol) and the solution was slowly warmed up to rt and stirred for 2h at the same temperature. The reaction mixture was slowly poured into crushed ice and extracted with  $\text{CH}_2\text{Cl}_2$ , washed with 5%  $\text{NaHCO}_3$ , water and brine and dried. Residue obtained after evaporation of the solvent was purified by silica gel column chromatography using 1:1 Hexane/ethyl acetate containing 2% TEA to afford compound 5 in 70% yield.  $^1\text{H}$  NMR  $\text{DMSO}-d_6$  1.03 - 1.12 (2d, 24H), 2.69 - 2.75 (2t, 4H), 3.1 - 3.33 (m, 4H), 3.33 - 3.55 (m, 5H), 3.66 - 3.7 (m, 4H), 3.72 (s, 6H), 6.83 - 6.89 (m, 4H), 7.19 - 7.48 (m, 9H).  $^{31}\text{P}$   $\text{D}_3\text{PO}_4$  as an external standard 153.64 & 153.39 (2S)

### Preparation of VEGF Dimers

Synthesis of VEGF dimers were done on 8800 automated DNA/RNA synthesizer. NX31838 was prepared, where rA stands for adenosine, mG and mA stands for 2'-O-methylguanosine and adenosine respectively and fC and fU stands for 2'-deoxy-2'-fluorocytidine and 2'-fluorouridine respectively and [3'-3'] stands for a 3',3'-internucleotidic linkage. The synthesis was carried out at a 1 mmol scale on a Millipore 8800 automated synthesizer using 5'-DMT-2'-O-methyl-N<sup>6</sup>-tert-butylphenoxyacetyl-adenosine, 5'-DMT-2'-O-TBDMS-N<sup>2</sup>-tert-butyl-phenoxyacetylguanosine and 5'-DMT-2'-O-TBDMS-N<sup>6</sup>-tert-butylphenoxyacetyl-adenosine 3'-N,N-diisopropyl-(2-cyanoethyl) phosphoramidites and 2'-deoxy-2'-fluoro-5'-DMT-N<sup>4</sup>-acetylcytidine and 2'-deoxy-2'-

fluoro-5'-DMT-uridine 3'-N,N-diisopropyl-(2-cyanoethyl)-phosphoramidites. The synthesis cycle was as follows. The activator formulations are described in the table. The syntheses were carried out using CPG support of 600 Å pore size, 80 - 120 mesh, and 60 - 70 µmol/g loading with 5'-succinylthymidine. The coupling cycle is shown in Table

5 12.

**Example 6. Pharmacokinetic Properties of phospholipid (PL) and PEG modified VEGF Nucleic Acid Ligands.**

10 Of the Sequences shown in Table 2, Sequence VT30.44 was chosen for further study and was renamed as NX31838. The pharmacokinetic properties of VEGF Nucleic Acid Ligand NX31838 conjugated to 20 and 40K PEG, were determined in Sprague Dawley rats (see Figure 1 for molecular descriptions) (SEQ ID NOS: ). Similar studies were also carried out on NX31838 conjugated to PL lipid as a liposomal  
15 formulation and as free drug (see Figure 1 for molecular descriptions) (SEQ ID NOS: ). In each study the oligonucleotide was diluted in PBS to a solution concentration of 1.0 mg/ml based on UV absorption at 260 nm and an extinction coefficient of 0.037 µg oligo/ml. In all studies, 9 rats received 1.0 mg oligonucleotide/kg animal weight by bolus tail vein injection and plasma samples were taken at various times from 2 minutes  
20 to 24 hours. The plasma samples and quality control samples were analyzed using a hybridization assay. The hybridization assay utilized a capture oligonucleotide that contains a complementary sequence to the 5'-end of the VEGF Nucleic Acid Ligand conjugated to an iron oxide (FeO) bead (FeO-spacer-3'-d (GCC TTA GTC ACT T-5') (SEQ ID NO: ) where spacer = (dT)<sub>8</sub>), and a detection oligonucleotide containing two  
25 biotin molecules at the 5'-end (biotin-biotin-5'-d(spacer-CGG ATG TAT AAG CA-3'), where spacer = (dT)<sub>8</sub>) (SEQ ID NO: ). After incubation of the capture and detect probes with a plasma sample containing VEGF Nucleic Acid Ligand NX31838 the amount of the biotin oligonucleotide hybridized to the bead was quantitated with the streptavidin-linked alkaline phosphatase, using CSPD-Sapphire as the luminescent substrate.

Data for the plasma concentration of the free, PEG20K and PEG40K VEGF Nucleic Acid Ligands (NX31838) (SEQ ID NOS: ) as a function of time following bolus injection are summarized in **Figure 6**. The 40K PEG conjugate was cleared with a monoexponential  $t_{1/2}$  of 360 minutes, while the 20K PEG version was cleared much more rapidly with 95% of the Nucleic Acid Ligand being cleared with an alpha  $t_{1/2}$  of 49 minutes and 5% being cleared with a beta  $t_{1/2}$  of 192 minutes, indicating the apparent importance of size on clearance. Compared with the PEG-conjugated Nucleic Acid Ligands, the free (unconjugated) NX31838 was cleared from plasma very rapidly with a  $t_{1/2}$  of several minutes. The plasma concentration of an oligonucleotide as a function of time can be significantly increased by introducing appropriate functional groups into the oligonucleotide.

Data for the plasma concentration of PL lipid conjugated VEGF Nucleic Acid Ligand (SEQ ID NOS: ) formulated with and without liposomes as a function of time following bolus injection are summarized in **Figure 7**. The liposomes were created as described in Example 7A by sonication in the presence of Nucleic Acid Ligand and contain oligonucleotide on the inside as well as the outside. The liposomal formulation was cleared much more slowly than the free drug, beta  $t_{1/2}$  of 1161 minutes and 131 minutes, respectively. The plasma concentration of an oligonucleotide as a function of time can be significantly increased by liposomal formulation.

20

### **Example 7. Preparation of NX31838 PL-Liposome Complex**

#### **A. Liposome preparation by filming.**

The lipids are combined at a ratio of 2 moles DSPC to 1 mole cholesterol. NX 31838 PL, in water, is added to the lipids at a ratio of 1:50 (w/w). The material is combined by solvating with a solution of chloroform : methanol : water (1:3:1). The solvent is removed by rotary evaporation leaving a heterogeneous film of NX 31838 PL co-mixed with the lipids. The film is rehydrated to 50 mg/mL, based on the lipids, in a solution of 9 % sucrose, buffered with 25 mM sodium phosphate at pH 7.4. The solution is mixed vigorously, heated to 65 °C and the resultant white milk-like solution

30

sonicated in 75 mL aliquots to assemble the lipids into unilamellar liposomes. The progress of liposome formation is followed visually until the solution becomes opalescent and then by particle sizing via dynamic light scattering using a particle analyzer (Leeds & Northrup Model Microtrack UPA 150, Horsham, PA). Liposome size is in the range of  
5 50 to 70 nm (by volume weight distribution method).

B. Liposome preparation by passive anchoring.

scNX-278 (see **Figure 1C** for molecular description) was tested to see whether it would undergo spontaneous incorporation into pre-formed ("empty") liposomes.

10 Preliminary results using a DEAE assay (for removal of free Nucleic Acid Ligand/glycerol lipid complex) indicated two important findings: 1) loading could be achieved; and, more importantly, 2) essentially complete loading of the Nucleic Acid Ligand/glycerol lipid complex was observed over 24 hours at room temperature. A more detailed study to determine the effects of temperature upon loading was subsequently  
15 undertaken. It was observed that temperature had a dramatic effect on the rates of incorporation. Although complete loading could be achieved over 24 hours at room temperature, complete incorporation could be achieved in just minutes at elevated temperatures (67°C). This proved to be a rapid and efficient method for incorporating Nucleic Acid Ligand/Lipophilic Compound Complex into pre-formed liposomes.

20 Size-exclusion chromatography was then used to separate free scNX-278 from the liposome-associated form. The preliminary work was conducted using the loading of scNX-278 into "empty" 2:1 DSPC:cholesterol liposomes. Chromatograms were generated using a Superdex S-200 column at 22°C. Over a 22 hour period, the gradual incorporation of the scNX-278 into the empty liposome population was observed as a  
25 shift in the peak areas (data not shown). The results correlate well with the data obtained from the DEAE assay.

Studies were also undertaken to determine whether additional scNX-278 could be loaded into sonicated oligo-liposomes. A sonicated preparation of scNX-278 was prepared by co-dissolving oligo-lipid with lipid and co-sonicating the two together. The  
30 resulting liposomes showed complete incorporation of the scNX-278. This sonicated

preparation was then subjected to 2 separate rounds of passive anchoring with additional free scNX-278 to see whether more scNX-278 could be incorporated successfully.

During the first round of passive anchoring, all of the free scNX-278 was passively anchored into the liposomes after incubation for 1 hour at 65°C. The second attempt at  
5 passive anchoring of additional scNX-278 resulted in incomplete loading.

The key finding from these experiments is that a Nucleic Acid Ligand/Lipophilic Compound Complex could be passively anchored into sonicated oligo-liposomes at high concentrations, but that the liposome's capacity for absorbing additional Nucleic Acid Ligand/Lipophilic Compound Complexes could be exceeded. After 2 rounds of passive  
10 loading (to approx. 3mg lipid-oligo/50 mg lipid), the liposomes apparently reach their "capacity" to absorb additional oligo-lipid since some free lipid-oligo remains. These data were confirmed by DEAE spin-column analysis (data not shown). The conclusions that can be drawn are: 1) sonicated liposomes possess additional capacity for incorporating Nucleic Acid Ligand/Lipophilic Compound Complexes; and 2) 100%  
15 Nucleic Acid Ligand incorporation can be achieved via sonication.

Subsequent studies were conducted on NX31838 PL (see **Figure 1E** for molecular description). NX31838 is of significant interest because it has improved pharmacokinetics (see Example 6) and biodistribution against VEGF targets when incorporated in liposomes. Several studies were conducted to better understand the  
20 incorporation of NX31838 via passive anchoring into liposomes.

Studies on the kinetics of NX31838 PL indicated that the passive anchoring for this molecule was so rapid as to be considered impossible to measure via any of the chromatography techniques known in the literature (all which require a minimum of several minutes of run time).

25 In order to determine the orientation of the NX31838 PL molecule (i.e., whether the Nucleic Acid Ligand component was projecting externally from the Liposome, or projecting into the Liposome aqueous center), externally introduced RNase was used to selectively cleave any of the Nucleic Acid Ligand Component that was projecting externally from the Liposome. In the case of passively anchored NX31838 PL  
30 liposomes, all of the Nucleic Acid Ligand is exposed to RNase I. No additional digestion

was observed following Triton X-100 treatment. These results indicate that the passively loaded NX31838 PL is oriented such that the Nucleic Acid Ligand Component is projecting externally from the Liposome. If the passively anchored NX31838 PL liposomes are pre-digested with RNase I, then run over a DEAE column approximately 5 99% of the Nucleic Acid Ligand is captured by the column, whereas if the same sample is run over DEAE but without pre-incubation with RNase I, nearly 100% of the oligo is able to pass through the column, unbound to the DEAE. Liposome protects the oligo from DEAE. The Liposome acts to protect the Nucleic Acid Ligand Component from DEAE, since it associates with the Nucleic Acid Ligand with high affinity, greatly 10 reducing its exposure to the DEAE groups.

Finally, as part of developing new methods to separate free Nucleic Acid Ligand/Lipophilic Compound Complex from the liposome-anchored form, we digested NX31838.05 PL with RNase I. The cleaved oligo could be easily separated using size exclusion chromatography (S-1000 resin) following removal of the lipid tail, whereas the 15 intact Nucleic Acid Ligand/Lipophilic Compound Complex co-eluted with liposomes under identical conditions. This data indicates that the Nucleic Acid Ligand/Lipophilic Compound Complex is probably forming a micelle when free in solution. This results in it co-eluting in the void volume of the column with the liposomes. Removal of the lipid tail allows it to enter the gel filtration media and hence be sized and stored appropriately.

20

#### **Example 8. In Vivo Efficacy of VEGF Nucleic Acid Ligand Complexes - Dermal Vascular Permeability Assay**

The ability of several different formulations of the NX31838 Nucleic Acid Ligand 25 to attenuate VEGF-induced changes in the permeability of the dermal vasculature (Miles Assay) was performed as previously described (Senger *et al.* (1986) Cancer Research 46:5629-5632) with minor modifications. Briefly, adult female guinea pigs (3/study) were anesthetized with isoflurane and the hair on the dorsal and lateral back areas was removed with clippers. Evans Blue dye (2.5 mg/guinea pig) was administered



intravenously. Injection solutions (PBS, VEGF, NX31838 formulations, and anti-VEGF monoclonal antibody) were prepared 30 min in advance, co-mixed where indicated, with final concentrations as shown. Each solution shown was then injected intradermally (duplicate injections/guinea pig; 40  $\mu$ l/site) in a randomized manner in a grid pattern drawn on the clipped area. Guinea pigs were allowed to recover from anesthesia and were sacrificed by CO<sub>2</sub> exposure 30 min after completion of the intradermal injections. The skin was then harvested, trimmed free of subcutis, and transilluminated. Images were then captured using a color CCD camera (Hitachi Denshi KP-50U, Japan) and Image-Pro Plus software (Version 3.1, Media Cybernetics, Silver Springs, MD). Each skin sample was normalized for intensity with each injection site analyzed for optical density and the area involved.

Panels A-C from **Figure 8** show the results of Nucleic Acid Ligand attenuation of VEGF-induced vascular leakage for NX31838-20K PEG, NX31838-40K PEG, NX31838-PL in liposomal preparation as described in Example 7A. All formulations were able to significantly reduce vascular leakage down to or near PBS control levels with concentrations as low as 100 nM. At 30 nM the blocking effect of the Nucleic Acid Ligand was lost. The NX31838-PL liposomal formulation was not evaluated at this concentration but appeared to have reduced blocking activity at 100 nM. The anti-VEGF monoclonal antibody was also evaluated in this model system (data not shown) and was likewise effective down through 100 nM with loss of activity at 30 nM. Thus, suggesting that in this model system that NX31838 in the various formulations examined is equally effective as antibody in blocking one of the functional effects of VEGF protein.

#### **Example 9. In Vivo Efficacy of VEGF Nucleic Acid Ligand Complexes - Corneal Pocket Model**

VEGF Nucleic Acid Ligand (NX31838) formulations were tested in their ability to reduce VEGF-induced corneal angiogenesis in the normally avascular rat cornea. Briefly, biopolymer (Hydron) pellets  $\pm$  VEGF protein (3 pmol) were prepared approximately 30 hr before by adding the protein or carrier solution to 12% biopolymer in 95% ethanol. Adult, Sprague-Dawley rats (200-240 g) were anesthetized by

- intraperitoneal injection of ketamine HCl (50 mg/kg) and xylazine (10 mg/kg). The left eye was then prepared by topical administration of tetracaine HCl for local anesthesia followed by application of dilute povidone-iodine solution and subsequent rinsing with isotonic saline solution. A vertical partial thickness incision was made in the mid-cornea.
- 5 A mid-stromal pocket was dissected caudally toward the lateral canthus extending to within 1.5 mm of the limbus. A pellet was then inserted into and pushed to the caudal limit of the pocket. Residual air was gently massaged out of the pocket. A drop of chloramphenicol ophthalmic solution was then applied to the eye. The animal was rolled over and the procedure repeated on the right eye with insertion of the same type of pellet.
- 10 Upon completion of pellet insertion in each eye, each animal was then administered either PBS (volume matched to Nucleic Acid Ligand formulation group) or Nucleic Acid Ligand (10 mg/kg) intravenously twice daily as indicated. At 5 days, each animal was anesthetized and photographs were taken using a 35 mm camera (Minolta X9) mounted on a dissecting microscope (KAPS, Germany). Each eye was evaluated for the angiogenic
- 15 response by measuring the maximum length of vessel growth (0-5), the density of vessel growth (1-4) adjacent to the implanted pellet, and the circumference of the eye with angiogenesis occurring (0-1). An angiogenic index was then determined as the product of length \* density \* circumference.

- The ability of Nucleic Acid Ligand formulations to block VEGF-induced
- 20 angiogenesis is seen in panels A-C in **Figure 9**. Despite being equally effective as the other formulations in blocking vascular permeability changes, NX31838-20K PEG was ineffective at attenuating the angiogenic response in the normally avascular cornea. However, both NX31838-40K PEG and liposomal NX31838-PL significantly reduced the level of angiogenesis by 65-70%. It is presumed that these differences are attributable
- 25 to the respective pharmacokinetic profiles of the Nucleic Acid Ligands.

Statistical Analysis: Groups in the Miles assay and corneal angiogenesis models were compared using Rank ANOVA with Dunnett's comparisons.

#### **Example 10. In Vivo Efficacy of VEGF Nucleic Acid Ligand in Tumor Models**

30

**Human Tumor Xenograft Model:** The ability of the VEGF Nucleic Acid Ligand NX31838 40K PEG to affect solid tumor growth was determined in a subcutaneous tumor model in nude mice. The A673 human rhabdomyosarcoma tumor cells were grown in tissue culture, harvested and  $1 \times 10^7$  viable cells were implanted subcutaneously, in nude mice, proximal to the axillary region of the flank. Treatment with test compounds was initiated 12 hours later, and continued for the duration of the experiment. Compounds were dosed intraperitoneally, twice daily at 10 and 40mg/kg. A negative control consisted of dosing a scrambled aptamer sequence, NX31917-40K PEG (See **Figure 1R** for molecular description) at 40 mg/kg twice daily, and a positive control consisted of anti-VEGF antibody Mab.26503.11 (R & D Systems, Lot # LDO3) dosed at 100µg/ mouse twice weekly. Both Nucleic Acid Ligand-treated groups, and the antibody treated groups demonstrated a significant slowing of tumor growth relative to the scrambled sequence negative control group (**Figure 11**). The % Tumor Growth Inhibition (TGI) , was determined to be 75% and 80% for the 40mg/kg and 10mg/kg BID groups and 83% for the monoclonal antibody treated group (**Table 8**). Since there appeared to be no significant difference between the 40mg/kg dose group and the 10mg/kg dose group, no further dosing of the 40mg/kg group occurred after day 14. As can be seen in **Figure 11**, several days after termination of dosing, tumors grew rapidly and mimicked the growth rate of the negative control group, while the 10mg/kg Nucleic Acid Ligand group and the antibody treated group continued to grow at a reduced rate.

Additional studies were performed using the same tumor model where new batches of VEGF Nucleic Acid Ligand, NX31838 40K PEG (designated NX31838.04 and NX31838.07) were compared, and also dose titrated downward from 10mg/kg BID, 3mg/kg BID and 1mg/kg BID. The experiment also included a once daily dose of 10mg/kg, as well as a Liposomal form of the VEGF Nucleic Acid Ligand, NX31838 PL at 10mg/kg BID. As can be seen in **Figure 12** and **Table 9**, the same degree of tumor growth inhibition was achieved in both experiments. Both batches of VEGF Nucleic Acid Ligand were equivalent when compared on the twice daily dosing schedule, with TGI values of 61% and 70% for the old and new batch, respectively. In addition, it was

determined that the once daily dosing (SID) was as effective as the twice daily dosing. However, the titration scheme used in this experiment failed to reach a no effect dose.

A third experiment was performed where further downward titration of the VEGF Nucleic Acid Ligand was able to demonstrate a dose response relationship relative to tumor growth. In this experiment the VEGF Nucleic Acid Ligand was titrated down, approaching a no effect dose of 0.03mg/kg. The relative tumor growth inhibition can be seen in **Figure 13** and is summarized in **Table 10**.

In addition to the three unstaged tumor studies, a staged tumor study was prepared where the tumors were allowed to establish and reach 200 +/- 100 mm<sup>3</sup> prior to initiation of treatment with the VEGF Nucleic Acid Ligand. The dose groups of 10 mg/kg of NX31838 40K PEG and the 100µg twice weekly of mAb 26503 (R & D Systems) achieved 59% and 69% tumor growth inhibition respectively (**Figure 14, Table 11**). These collective studies demonstrate that the VEGF Nucleic Acid Ligand is able to slow the A673 tumor from establishing as well as inhibiting tumor growth once tumors have established.

**Kaposi's Sarcoma Model:** The effect of NX 31838-40 K PEG on the subcutaneous growth of Kaposi's Sarcoma cell line KSY-1 in nude mice was also examined. KSY-1 cells are unique among tumor cell lines in that they can be inhibited in culture by VEGF antagonists. KSY-1 cells were grown in culture, pooled and injected subcutaneously (2x10<sup>7</sup> cells/mouse) in the hind flank of mice. Three groups of mice (4 mice per group) were treated by intraperitoneal injections every 12 hours with either 30 mg/kg of NX31838-40 K PEG, 30 mg/kg NX31917-40 K PEG (see **Figure 1R** for molecular description) or PBS for the duration of the experiment. Treatment was initiated one day after tumor cell implantation. While tumor growth in the PBS-treated and NX31917-40 K PEG-treated groups was comparable, considerable inhibition of tumor growth was observed in the NX31838-40 K PEG-treated group (**Figure 16**). NX31838-40 K PEG inhibited the growth of KSY-1 tumors by 65% (compared with the PBS-treated group) or by 69% (compared with the NX31917-40 K PEG-treated group) at the time the experiment was terminated (day 22).

**Example 11. Intravitreal Pharmacokinetics of VEGF Nucleic Acid Ligand  
NX31838+40KPEG in Rabbits**

- New Zealand White rabbits were treated with VEGF Nucleic Acid Ligand NX31838 conjugated to 40mPEG by intravitreal administration at a dose of 0.5 mg/eye.
- 5 40K PEG was conjugated to the VEGF Nucleic Acid Ligand as described in Example 5, and the resulting complex is as shown in **Figure 1H** (SEQ ID NO: ). Rabbits received intravitreal injection of NX31838-40K PEG in each eye. The time between doses for a given animal did not exceed 15 minutes. Blood and vitreous samples were collected as specified in **Table 7**.
- 10 Analysis of plasma and vitreous samples were carried out by the use of a double hybridization assay. In this assay, two hybridization probes are used, a capture probe attached to wells of 96 well plates, and a biotinylated detect probe. The capture probe forms a hybrid with the 5' end of the Nucleic Acid Ligand. This assay is highly specific and sensitive to full length Nucleic Acid Ligand to yield a positive signal. The current
- 15 limit of quantitation is approximately 2 fmoles in 5  $\mu$ l of plasma.

**Table 1.** Summary of VEGF Nucleic Acid Ligand pharmacokinetic parameters after i. v. bolus administration in Sprague Dawley rats determined from the data shown in **Figure 15** (compartmental analysis).

<u>Parameter</u>	<u>NX213</u>	<u>NX278</u>	<u>NX278-L</u>
Total AUC ( $\mu\text{g}\cdot\text{min}/\text{ml}$ )	147	202	531
C, t=0 min ( $\mu\text{g}/\text{ml}$ )	14.59	23.16	16.95
C, t=2 min ( $\mu\text{g}/\text{ml}$ )	15.31	14.08	15.74
$\alpha t_{1/2}$ (min)	7	3	13
$\beta t_{1/2}$ (min)	49	67	113
Clearance ( $\text{ml}/\text{kg}/\text{min}$ )	6.80	4.95	1.88
$v_{ss}$ ( $\text{ml}/\text{kg}$ )	72	251	152

Table 2. 2'-F-pyrimidine ligands to VEGF<sub>165</sub>

Ligand (frequency)	5'-gggaaggacgaugcagg [variable region] cagacgaucucgcccga-3'	Sequence of variable region	K <sub>d</sub> (nM)
Family 1			
VP30.7	gcggg	gAAGAAUUGG	3000
VP30.12	gcggAAUACG	GAAGAAUUGG	7
VP30.13 (7)	ugcgggGAUACA	GAAGAAUUGG	10
VP30.16	AUGAUCCGCGUAG	GAAAGUAUUGG	6
VP30.19	gcggCACUUVAA	GAAGAAUUGA	9
VP30.22 (6)	gcggUAG	GAAGAAUUGG	20
VP30.25	cggcCGGGAUUUUG	GAAGAAUUGG	20
VP30.26 (2)	gcggCGGYACUUUG	GAAGAAUUGA	10
VP30.27	gcggg	GAAGAAUUGG	400
VP30.40	ugcgAAACG	GAAGAAUUGG	6
VP30.41	gcggUAG	GAAGUUUUGU	7
VP30.51 (2)	gcggAGUUUUUG	GAAGAAUUGG	90
VP30.54	gcggAAGAAACG	GAAGAAUUGG	10
VP40.4 (5)	gggaaggacgaugcgg	GAAGAAUUGA	200
VP40.43	gggaaggacgaugcggACA	GAAGAAUUGG	30
VP40.53	ugcgguUGAGAGAAACG	GAAGAAUUGG	8
VT30.4	augcgguCUUAAAGUUUUG	GAAGAAUUGA	20
VT30.7	gcggUUAAACCAAGUG	GAAGAAUUGG	10
VT30.10	augcgguAAACG	GAAGAAUUGG	2
VT30.13	ugcgguCAGGAUUUUUG	GAAGAAUUGG	10
VT30.20	gaugcgguAAACG	GAAGAAUUGG	4
VT30.52	ugcggg	GAAGAAUUGA	9000
VT30.53	gaugcgguAGCUAAACG	GAAGAAUUGG	10

Ligand	Sequence of variable region		Kd
(frequency)	5' - gggaggacgaugcgg [variable region] caagacacucgccccga - 3'	(pM)	
Family 2			
VP30.2 (5)	ggyGA ACCGA UGGA UUU UUGGACGC UCGCCU	10	
VP30.5 (4)	gAYCA ACCGA UUGAC GUUA UGGACGC UGGUC	8	
VP30.31 (5)	gcgGUU ACCGA UUGAA CUUC UUGACGC UACCGU	6	
VP30.43	gGUU ACCGA UUGAA GUUA UUGACGC UACCU	5	
VP40.9	gGAGCAGA ACCGA UAGAA GAA UUGACGC UCAUCUCCGGU	30	
VP40.14	GUACCAGAUAGCA ACCGA AUGAA GAA CUGGACGC UGCUca	8	
VP40.17	ugcgGUU GA ACCGA UGGAA UCGC UUGACGC UCAUCCGCA CGUUGCU	10	
VT30.9 (6)	gGUCA ACCGG UUGAA UAU UUGGUCGC UGACCU	30	
Family 3			
VT30.1 (2)	gacgaugcgg A ACUA GUGAUGCUU AUA CGA CCGUGUUGUC	10	
VT30.2	gcgg AVCA GUGAUGCUU AUA GA CCGCCUCCGU	2	
VT30.3 (7)	gaugcgg AGA AUCA GUGAUGCUU AUA AAUC UCGYUC	5	
VT30.11	gaugcgg A AVCA GUGAUGCUU AUA GCUC CCGCGUCCU	4	
VT30.15	gcgg A ACCA GUGAUGCUU AUA AGA CUGGUCGU	3	
VT30.21	cgaugcgg AVCA GUGAUGCUU AUA GA CCGUAUUGCGU	6	
VT30.28	gaugcgg AGA AUCA GUGAUGCUU AUA AACC UCGUGUC	60	
VT30.29	augcgg AVCA GUGAUGCUU AUA GC UCCGCGUGGU	10	
VT30.35	cgg ACCA GUGAUGCUU AUA AGCCCA UCGACCU	N.D.	
VT30.41	gaugcgg CAGG GUGAUGCCA AUG UACUUU UCGCGUC	40	
VT30.42	gacgaugcgg AVCA GUGAUGCUU AUA GC UCCACGUCGUC	N.D.	
VT30.44	gcgg AVCA GUGAUGCUU AUA CA UCCGCUCCGU	10	
VT30.54	gcgg ACUAG GUGAUGCCA AUA UUCUUU UCCGU	10	



Table 3.

Ligand	Sequence	Length (nts)	K <sub>D</sub> (pM)
t22	GACGAUGC <sup>CG</sup> GUAGGAAGAAUUGGAAGCGC*	29	70
t22a	GACGAUGC <sup>CG</sup> GUAGGAAGAAUUGGAAGCG	28	3000
t22b	ACGAUGC <sup>CG</sup> GUAGGAAGAAUUGGAAGCG <u>C</u>	28	80
t22c	GCGGUAGGAAGAAUUGGAAGCG <u>C</u>	23	90
t22d	CGGUAGGAAGAAUUGGAAGCG <u>C</u>	22	100
t22e	GGUAGGAAGAAUUGGAAGCGC*	21	200
t22f	GUAGGAAGAAUUGGAAGCGC*	20	>100,000
t2	GGCGAACCGAUGGAAUUUUUGGACGCUCGCC*	31	20
t2a	GCGAACCGAUGGAAUUUUUGGACGCUC <u>CG</u>	29	40
t2b	CGAACCGAUGGAAUUUUUGGACGCUCG	27	100
t2c	GAACCGAUGGAAUUUUUGGACGCUC*	25	200
t2d	AACCGAUGGAAUUUUUGGACGCU*	23	20,000
t2e	ACCGAUGGAAUUUUUGGACGC*	21	>100,000
t44	GCGGAAUCAGUGAAUGCUUAUACAUCCGC*	29	10
t44a	CGGAAUCAGUGAAUGCUUAUACAUCG	27	10
t44b	GGAAUCAGUGAAUGCUUAUACA <u>UC</u>	25	60
t44c	GAAUCAGUGAAUGCUUAUACAUC*	23	2000
t44d	AAUCAGUGAAUGCUUAUACA*	21	>100,000
t44e	AUCAGUGAAUGCUUAUACA*	19	>100,000

Table 4. Effect of 2'-OMe-purine substitutions on affinity for VEGF.

Ligand	Sequence	K <sub>D</sub> (pM)
t22OMe (OH-10,12,22)	GACGAUGCGGUAGGAAGAAUUGGAAGCGC	10
t22OMe (OH-10,12)	GACGAUGCGGUAGGAAGAAUUGGAAGCGC	20
t22OMe (OH-10,22)	GACGAUGCGGUAGGAAGAAUUGGAAGCGC	4,000
t22OMe (OH-12,22)	GACGAUGCGGUAGGAAGAAUUGGAAGCGC	90
t2OMe (OH-6,21)	GGCGAACC GAUGGAAUUUUUGGACGCUCGCC	60
t2OMe (OH-6)	GGCGAACC GAUGGAAUUUUUGGACGCUCGCC	500
t2OMe (OH-21)	GGCGAACC GAUGGAAUUUUUGGACGCUCGCC	20,000
t44OMe (OH-5,6)	GCGGA <del>A</del> UCAGUGAAUGCUUAUACAUC CGC	40
t44OMe (OH-5)	GCGGA <del>A</del> UCAGUGAAUGCUUAUACAUC CGC	>100,000
t44OMe (OH-6)	GCGGA <del>A</del> UCAGUGAAUGCUUAUACAUC CGC	>100,000

Table 5.

Ligand	Sequence	$K_D$ (s.d.) (pM)	$k_d$ (s.d.) (sec <sup>-1</sup> )	$k_a$ (M <sup>-1</sup> sec <sup>-1</sup> )
t22OMe	GCGGUAGGAAGAAUUGGAAGCGC	67 (36)	0.012 (0.004)	$1.8 \times 10^8$
t2OMe	GCGA <del>A</del> CCGAUGGAAUUUUUGGACGCUCGC	140 (50)	0.0042 (0.002)	$3.0 \times 10^7$
t44OMe	CGG <del>A</del> AUCAGUGAAUGCUUAUACAUCG	51 (11)	0.0074 (0.002)	$1.5 \times 10^8$

Table 6. Additional 2'-F-pyrimidine ligands to VEGF<sub>165</sub>.

Ligand (frequency)	Sequence of variable region 5' -gggaggacgaugcgg [variable region] cagacgacucgcccga -3'	Kd (pM)
VP30.1	TCTTTGAGTTTTTGCCAACGGTTTTCGCT	32,000
VP30.6	AACGGAATTCTTGGATACACACCTCGTCCT	20
VP30.11	TCAGGAACGGAATTTTTGGAGACACGCCCT	25
VP30.14	ACTGGGAGAATCCGAAAAACCTTCACGCGT	25
VP30.18	ATCCATCATTTAACC GTTTGCTCTCCCCCT	27
VP30.20 (3)	TTGATCGGACGTTAGTCATTTCCCGATCGT	57
VP30.23	GAGCTTGAAGTTTCAGTATTGGCACAACCT	63
VP30.29	CGCCACTTTGGAAGTTATTGAATTTTCGCGT	7
VP30.35	TGAATGAGCTGACGACCCTGAATTGCTCGT	6
VP30.48	GAGCTTGAAGTTTCGGTATTGGCACAACCT	>10,000
VP30.58	CAACTATTCGTTGATGTTTCCGTGAGCCGT	6
VP30.61	GAGCTTGAAGTTTCAGTACTGGCACAACCT	43
VP30.63	AACCAATAGAGATCTTCGGCTGCCCCGCGT	16
VP30.65	AAAACGCTTTTCTTGGCCCCCTCGTTGCGC	33
VP30.67	TTAACGGAATTCTTGGATACATAGCATGGT	24
VP40.1	CAAAGTTTGAGTTGATCTGATACGTTTCAGTATTGGCGT	N. D.
VP40.2 (5)	ATCTGTGAACTGGGTTTTTGCCGACGGTTACGCTTTTGCT	35
VP40.3 (5)	CAAAAGTTTGAGTTGATCTGATACGTTTCAGTATTGGCGT	2,000
VP40.5	TTGATCGAGGTTCTAAAGCCTATTTCCCTGACTTTCTCCCC	19
VP40.10	ATCTGTGAACTGGGTTTTTGCCGACGGTTACGCTTTTGCT	N. D.
VP40.11 (6)	AAGGAAGATGTTGATCGTTTGACGTGATGTGGATCCGCGT	980
VP40.18	TAGTAAGTTATTGAAAGCGCATCTCTATCAACTCTCGGCC	12
VP40.20	TACTTTCTTCTTTCTTTGCCTTTCTTTTCTTTTACGCCT	N. D.
VP40.21	CAGTTAATTAATTTGAGTTGTGATGTGTGTCGTTATGGGT	>100,000
VP40.24	GATGCTGAGTGAGGAAGTCTGATTGTTGCAGTATTGGCGT	5,000
VP40.25	AATGGAATTTGAGTCGATCTAGAATGCGTCGTATGGGCT	740
VP40.26	ACTCAACTGGACGCTATGTTGACGGTTATCGCTTTTGGGT	13
VP40.36	CAGGTTCAAGATTGGCAGTCGCATTGATCTTTTTCACCGC	1,300
VP40.37	CAAAAGTTTGAGTTGATCTGATACGTTTCCAGTATTGGCGT	N. D.
VP40.39	CAGTTAATTAAGTTGAGTTGTGATGTGTGTCGTTATGGGT	73,000
VP40.41	CAAATTCAAGGTCGAGTTATGCGTAGATGTGGCTCCTGTG	11,000

Ligand (frequency)	Sequence of variable region 5' - <u>gggaggacgaugcgg</u> [variable region] <u>cagacgacucgcccga</u> - 3'	Kd (nM)
VP40.44	ATCTGTTGAACTGGGTTTGGCCGACGGTTACGCTTTTGCT	43
VP40.47	CAGTTAATTAATTTGAGTTGTGATGTGTGTCGTTATGGGC	96
VT30.8	TTGATCGATTTTCCTGGCGTCCTTATGGGT	34
VT30.12 (4)	TCTTTGGGTTTTTGCCAACGGTTTTTCGCT	9
VT30.18	TTCAGAATTGGCTGCGATCCTTTTCCCCCT	4
VT30.22	TTGATCGACTTTTCCTGATCTTCTCCTCCT	N. D.
VT30.24	CACTAGGTGCATGCCATGAAATCTTGCTGT	N. D.
VT30.27	GATCACGGCTTTGCACGATCTTCTTCTCCT	120
VT30.23	GATCACGATACTTGACGATTTTCCTCTCCT	19
VT30.38	AGCGGTATTCTGTTTCGGTCGTTTTCTCCT	5
VT30.40	ATTTGGATGCATGTCAAGGCGTTTTGCCCT	30

Table 7: Group Assignments and Sample Times

(Study #1)

Group #	# of Rabbits	Blood Sample Time	Termination (Vitreous Sample Time)
1	2	predose, 15 min, 30 min, 1 hrs,	1 hours
2	2	predose, 30 min, 1 hr, 2 hrs, 6 hrs,	6 hours
3	2	predose, 1 hr, 4 hrs, 8 hrs, 24 hrs	24 hours
4	2	predose, 15 min, 6 hrs, 8 hrs, 24 hrs, 48 hrs 72 hrs	72 hours
5	2	predose, 2 hr, 4 hrs, 4 days, 5 days, 7 days	7 days

(Study #2)

Group #	# of Rabbits	Blood Sample Time	Termination (Vitreous Sample Time)
1	2	24 hrs, 7 days	7 days
2	2	24 hrs, 7 days, 13 days	13 days
3	2	24 hrs, 7 days, 13 days, 21 days, 28 days	28 days

**Table 8**  
**Anti-Tumor Efficacy of VEGF Nucleic**  
**Acid Ligand (NX31838) in the A673 Nude Mouse Xenograph Model**

**% Tumor Growth Inhibition (TGI)**

<b>Treatment</b>	<b>Mean Tumor Volume</b>	<b>Day</b>	<b>%TGI</b>
Scrambled Nucleic Acid Ligand 40 mg/kg BID	2823	13	0
VEGF Nucleic Acid Ligand 40 mg/kg BID	710	13	74.8
VEGF Nucleic Acid Ligand 10 mg/kg BID	565	13	80.0
Anti-VEGF mAb 2X weekly	489	13	82.7

%TGI =  $100(1 - W_t/W_c)$ ;  $W_t$  is the mean tumor volume of the treated group at time x;  $W_c$  is the mean tumor volume of the control group at time x

42% TGI or greater is significant

<b><u>Tumor Growth Delay</u></b>	<b>Day to 1000 mm<sup>3</sup></b>	<b>GD (t<sub>1</sub> - t<sub>2</sub>)</b>	<b>Day to 1500 mm<sup>3</sup></b>	<b>GD</b>
Scrambled Nucleic Acid Ligand 40 mg/kg BID	8.5	0	10	0
VEGF Nucleic Acid Ligand 40 mg/kg BID	14.5	6**	--	-
VEGF Nucleic Acid Ligand 10 mg/kg BID	17	8.5	20	10
Anti-VEGF mAb 2X weekly	18	9.5	21	11

**\*\*note: dosing for VEGF Nucleic Acid Ligand @ 40 mg/kg was terminated on day 14**

**Table 9**

**Anti-Tumor Efficacy of VEGF Nucleic Acid Ligand  
(NX31838) in the A673 Nude Mouse Xenograph Model**

**% Tumor Growth Inhibition (TGI)**

<b>Treatment</b>	<b>Mean Tumor Volume mean (<math>\pm</math> SD)</b>	<b># Animals</b>	<b>Day</b>	<b>%TGI</b>
PBS control	2357 ( $\pm$ 1361)	8	16	0
VEGF NX31838.04 10 mg/kg BID	930 ( $\pm$ 312)	7	16	61
VEGF NX31838.04 3 mg/kg BID	1135 ( $\pm$ 364)	7	16	52
VEGF NX31838.04 1 mg/kg BID	1045 ( $\pm$ 265)	8	16	56
VEGF NX31838.04 10 mg/kg SID	713 ( $\pm$ 206)	6	16	70
VEGF NX31838.07 10 mg/kg BID	570 ( $\pm$ 273)	6	16	76
VEGF NX31838PL 10 mg/kg BID	555 ( $\pm$ 174)	8	16	76

**%TGI = 100(1-W<sub>t</sub>/W<sub>c</sub>); W<sub>t</sub> is the mean tumor volume of the treated group at time x; W<sub>c</sub> is the mean tumor volume of the control group at time x**

**42% TGI or greater is significant**



**Table 10**  
**Anti-tumor Efficacy of VEGF Nucleic Acid Ligand**  
**(NX31838) 40K PEG in Nude Mouse Xenograph Model**

**% Tumor Growth Inhibition (TGI)**

<b>Treatment</b>	<b>Mean Tumor Volume Mean (<math>\pm</math>SD)</b>	<b># Animals</b>	<b>Day</b>	<b>%TGI</b>
PBS Control	3446( $\pm$ 1522)	8	14	0
NX31838 10mg/kg	540( $\pm$ 122)	8	14	84
NX31838 3mg/kg	795( $\pm$ 403)	7	14	77
NX31838 0.3mg/kg	1261( $\pm$ 337)	8	14	63
NX31838 0.03mg/kg	1773( $\pm$ 785)	8	14	49

$\%TGI=100(1-W_t/W_c)$ ;  $W_t$  is the mean tumor volume of the treated group at time x;  $W_c$  is the mean tumor volume of the control group at time x

42% TGI or greater is significant

**Table 11**

**Anti-Tumor Efficacy of VEGF Nucleic Acid Ligand  
(NX31838) vs Anti-VEGF MAb in a Growth Staged A673 Xenograph  
Model**

**% Tumor Growth Inhibition (TGI)**

<b>Treatment</b>	<b>Mean Tumor Volume Mean (<math>\pm</math>SD)</b>	<b># Animals</b>	<b>Day</b>	<b>%TGI</b>
PBS Control	3082( $\pm$ 1198)	8	12	0
NX31838 10mg/kg	1278( $\pm$ 543)	8	12	59
Anti-VEGF Mab 100 $\mu$ g Twice weekly	959( $\pm$ 359)	8	12	69

**%TGI=100(1-W<sub>t</sub>/W<sub>c</sub>); W<sub>t</sub> is the mean tumor volume of the treated group at time x; W<sub>c</sub> is the mean tumor volume of the control group at time x  
42% TGI or greater is significant**

**Tumor Growth Delay (TGD)**

<b>Treatment</b>	<b>Days to 1000mm<sup>3</sup></b>	<b>TGD(t<sub>t</sub>-t<sub>c</sub>)</b>	<b>Days to 2000 mm<sup>3</sup></b>	<b>TGD(t<sub>t</sub>-t<sub>c</sub>)</b>
PBS Control	6.3		9.5	
NX31838 10mg/kg	10	3.7	15.5	6
Anti-VEGF mAb 100 $\mu$ g Twice weekly	12.3	6	18.3	8.8

**Table 12.** Automated synthesis cycle for the preparation of NX31838

Step	Reagents	Equivalents*	Reaction Time
Detritylation	Dichloroacetic acid in CH <sub>2</sub> Cl <sub>2</sub> (3 % v/v)	250	15 min
Coupling	Nucleoside phosphoramidite (0.2M in CH <sub>3</sub> CN)	2	20min for fC & fU and 30min for all others
	1.0 M DCI	8**	
Oxidation	0.05 M I <sub>2</sub> in 2:1 pyridine: water	5.2	2
Capping	Cap A: 1:1:8 Ac <sub>2</sub> O:2,6- lutidine:THF Cap B: 16% NMI in THF	32	1

\*Equivalents are based on the moles of CPG-bound 3'-terminal nucleoside.

\*\* Activator equivalents are based on moles of nucleoside phosphoramidite.

General procedure for the synthesis of dimers

All other phosphoramidites were coupled by the same manner as mentioned above, except the glycerol bis amidite

Step	Reagents	Equivalents*	Reaction Time
Detritylation	Dichloroacetic acid in CH <sub>2</sub> Cl <sub>2</sub> (3 % v/v)	250	15 min
Coupling (Coupled twice)	Nucleoside phosphoramidite (0.04M in CH <sub>3</sub> CN)	0.75 eq per coupling	2 x 20min
	1.0 M DCI	16**	
Oxidation	0.05 M I <sub>2</sub> in 2:1 pyridine: water	5.2	2
Capping	Cap A: 1:1:8 Ac <sub>2</sub> O:2,6- lutidine:THF Cap B: 16% NMI in THF	32	1

**WE CLAIM:**

1. A purified and isolated non-naturally occurring RNA ligand to VEGF  
5 wherein said ligand is comprised of 2'fluoro (2'F)-modified nucleotides.
2. The RNA ligand to VEGF of claim 1 wherein said ligand is selected from the group consisting of the sequences set forth in Tables 2-6 (SEQ ID NOS: ).
- 10 3. The RNA ligand of claim 2 wherein said ligand is substantially homologous to and has substantially the same ability to bind VEGF as a ligand selected from the group consisting of the sequences set forth in Tables 2-6 (SEQ ID NOS: ).
4. The RNA ligand of claim 2 wherein said ligand has substantially the same  
15 structure and substantially the same ability to bind VEGF as a ligand selected from the group consisting of the sequences set forth in Tables 2-6 (SEQ ID NOS: ).
5. The RNA ligand to VEGF of claim 1 identified according to the method comprising:  
20 a) contacting a Candidate Mixture of RNA with VEGF, wherein the RNA having an increased affinity to VEGF relative to the Candidate Mixture may be partitioned from the remainder of the Candidate Mixture;  
b) partitioning the increased affinity RNA from the remainder of the Candidate Mixture; and  
25 c) amplifying the increased affinity RNA to yield a mixture of RNA enriched for RNA having an increased affinity for VEGF; whereby RNA Ligands of VEGF are produced.
6. The RNA ligand to VEGF of claim 2 identified according to the method  
30 comprising:  
a) contacting a Candidate Mixture of RNA with VEGF, wherein the RNA having an increased affinity to VEGF relative to the Candidate Mixture may be partitioned from the remainder of the Candidate Mixture;  
b) partitioning the increased affinity RNA from the remainder of the  
35 Candidate Mixture; and  
c) amplifying the increased affinity RNA to yield a mixture of RNA enriched for RNA having an increased affinity for VEGF; whereby RNA Ligands of VEGF are produced.
- 40 7. A Complex comprised of a VEGF Nucleic Acid Ligand comprising 2'F-modified nucleotides and a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound.
8. The Complex of Claim 7 further comprising a Linker between said Ligand  
45 and said Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound.

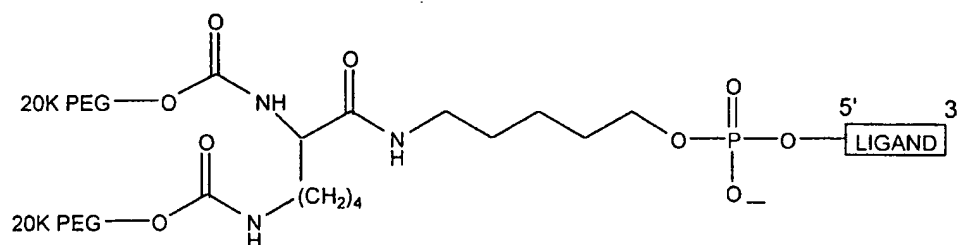
9. The Complex of Claim 7 wherein said Non-Immunogenic, High Molecular Weight Compound is a Polyalkylene Glycol.

5 10. The Complex of claim 9 wherein said Polyalkylene Glycol is polyethylene glycol.

10 11. The Complex of claim 10 wherein said polyethylene glycol has a molecular weight of about between 10-80 K.

12. The Complex of claim 10 wherein said polyethylene glycol has a molecular weight of about between 20-45 K.

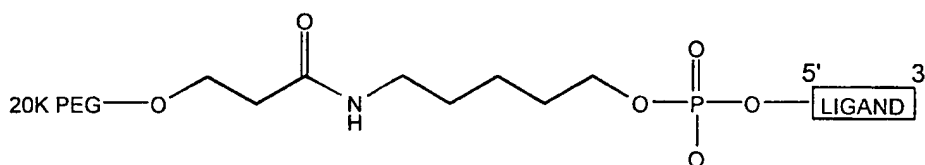
15 13. The Complex of claim 10 wherein said Complex is



Ligand Component =

fCmGmGrArAfUfCmAmGfUmGmAmAfUmGfCfUfUmAfUmAfCmAfUfCfCmG-3'3'-dT  
(VEGF ligand)

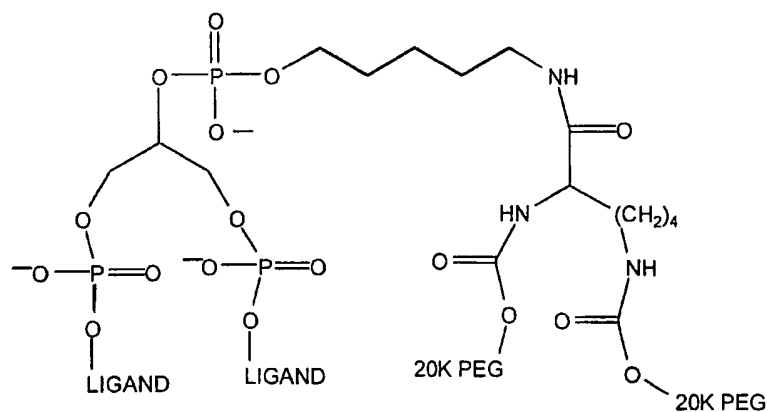
14. The Complex of claim 10 wherein said Complex is



Ligand Component =

20 fCmGmGrArAfUfCmAmGfUmGmAmAfUmGfCfUfUmAfUmAfCmAfUfCfCmG-3'3'-dT  
(VEGF ligand)

15. The Complex of claim 10 wherein said Complex is

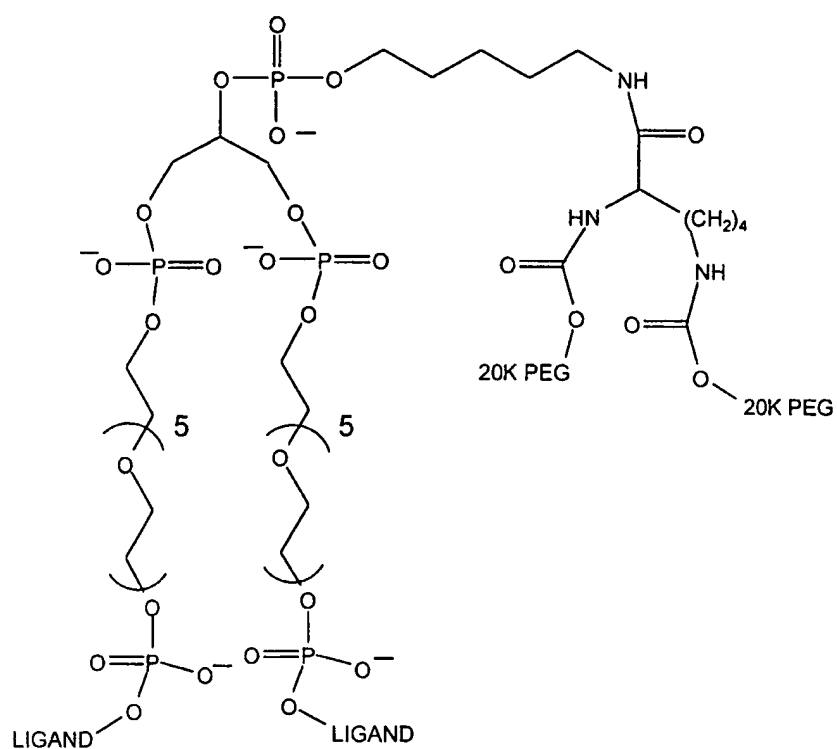


Ligand Component =

fCmGmGrArAfUfCmAmGfUmGmAmAfUmGfCfUfUmAfUmAfCmAfUfCfCmG-3'3'-dT  
(VEGF ligand)

5

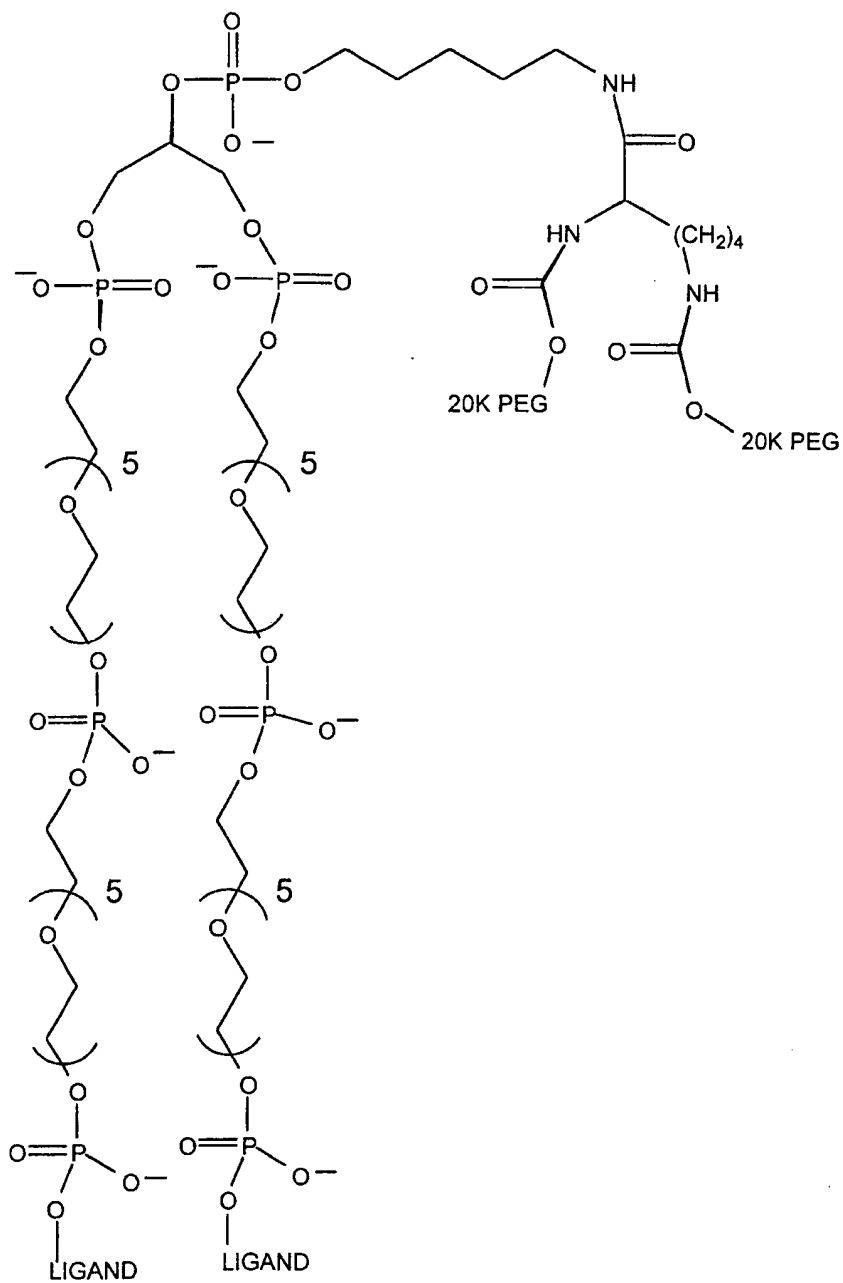
16. The Complex of claim 10 wherein said Complex is



Ligand Component =

fCmGmGrArAfUfCmAmGfUmGmAmAfUmGfCfUfUmAfUmAfCmAfUfCfCmG-3'3'-dT  
(VEGF ligand)

17. The Complex of claim 10 wherein said Complex is



Ligand Component =

fCmGmGrArAfUfCmAmGfUmGmAmAfUmGfCfUfUmAfUmAfCmAfUfCfCmG-3'3'-dT  
(VEGF ligand)



18. The Complex of claim 7 wherein said Lipophilic Compound is a glycerol lipid.

19. The Complex of claim 18 wherein said Lipophilic compound is a glycerol lipid having the structure



10 where  $R^1$ ,  $R^2$ , and  $R^3$  are independently selected from the group consisting of  $CH_3(CH_2)_n-O(PO_3)-CH_2-$ ; and  $CH_3(CH_2)_n-CONH_2-CH_2-$ ,  $CH_3(CH_2)_nO-$ ,  $CH_3(CH_2)_nOCH_2-$ ,  $CH_3(CH_2)_n(CO)OCH_2-$ ,  $CH_3(CH_2)_n(CO)O-$  and  $X-$ , wherein at least one must be  $X-$ , and  $X$  is independently selected from the group consisting of  $(PO_4)$ ,  $O$  and  $CH_2OC=O$ , and wherein  $n=0-30$ , preferably  $10-20$ .

15

20. The Complex of claim 19 wherein said glycerol lipid is a phospholipid wherein  $R$  is  $CH_3(CH_2)_n-O(PO_3)-CH_2-$ .

21. The Complex claim 20 wherein  $R^1$  is  $CH_3(CH_2)_n-O(PO_3)-CH_2-$ ,  
20  $R^2$  is  $CH_3(CH_2)_n-O(PO_3)-CH_2-$ , and  $R^3$  is  $X-$ , and wherein  $n = 17$ .

22. The Complex of claim 21 wherein  $X$  is  $PO_4$ .

23. The Complex of claim 19 wherein said glycerol lipid is a glycerol amide  
25 lipid wherein  $R$  is  $CH_3(CH_2)_n-CONH_2-CH_2-$ .

24. The Complex of claim 23 wherein  $R^1$  is  $CH_3(CH_2)_n-CONH_2-CH_2-$ ,  
 $R^2$  is  $CH_3(CH_2)_n-CONH_2-CH_2-$ , and  $R^3$  is  $X-$ , and wherein  $n = 16$ .

30 25. The Complex of claim 24 wherein  $X$  is  $PO_4$ .

26. The Complex of claim 24 wherein  $X$  is  $O$ .

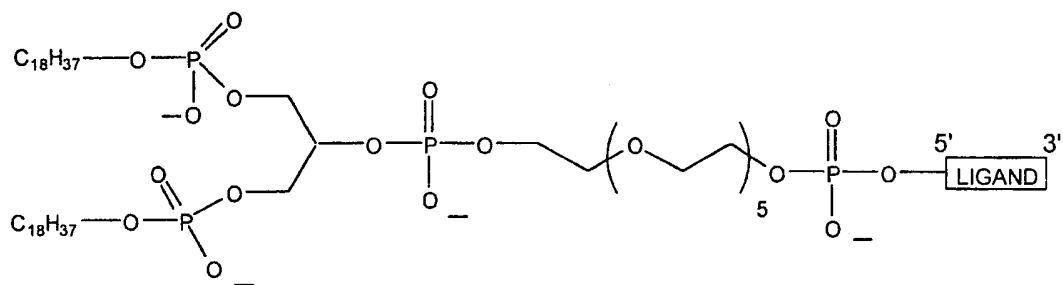
27. The Complex of claim 19 wherein  $R^1$  is  $CH_3(CH_2)_nO-$ ,  
35  $R^2$  is  $CH_3(CH_2)_nOCH_2-$ , and  $R^3$  is  $CH_2O(CO)$  and wherein  $n = 17$ .

28. The Complex of claim 19 wherein  $R^1$  is  $CH_3(CH_2)_n(CO)O-$ ,  
 $R^2$  is  $CH_3(CH_2)_n(CO)OCH_2-$ , and  $R^3$  is  $CH_2O(CO)$  and wherein  $n = 17$ .

40 29. A Lipid Construct comprising the Complex of claim 7.

30. A Lipid Construct comprising the Complex of claim 19.

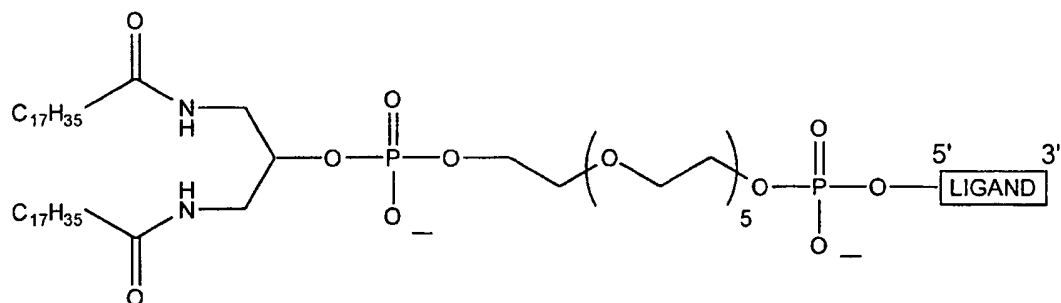
31. A Lipid Construct comprising the Complex of claim 20.
32. A Lipid Construct comprising the Complex of claim 21.
- 5 33. A Lipid Construct comprising the Complex of claim 22.
34. The Lipid Construct of claim 33 wherein said Complex is



Ligand Component =

fCmGmGrArAfUfCmAmGfUmGmAmAfUmGfCfUfUmAfUmAfCmAfUfCfCmG-3'3'-dT  
(VEGF ligand)

35. A Lipid Construct comprising the Complex of claim 23.
36. A Lipid Construct comprising the Complex of claim 24.
- 15 37. A Lipid Construct comprising the Complex of claim 25.
38. The Lipid Construct of claim 37 wherein said Complex is

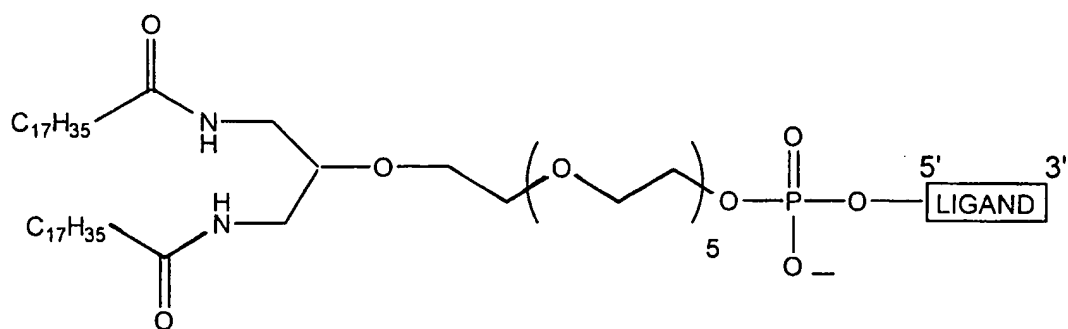


Ligand Component =

fCmGmGrArAfUfCmAmGfUmGmAmAfUmGfCfUfUmAfUmAfCmAfUfCfCmG-3'3'-dT  
(VEGF ligand)

39. A Lipid Construct comprising the Complex of claim 26.

40. The Lipid Construct of claim 39 wherein said Complex is



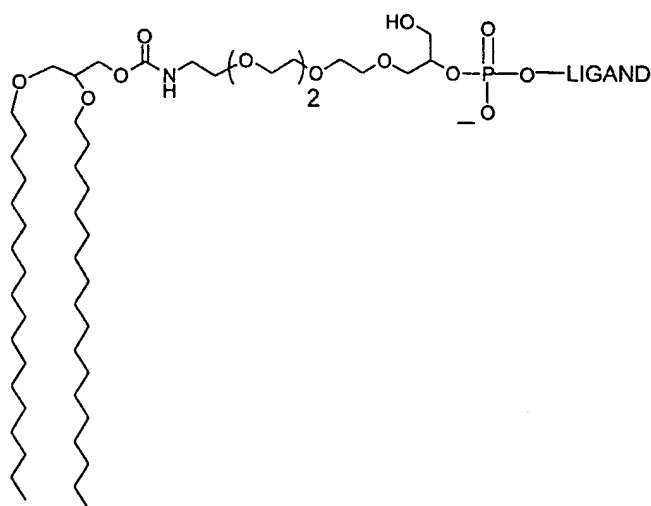
Ligand Component =

fCmGmGrArAfUfCmAmGfUmGmAmAfUmGfCfUfUmAfUmAfCmAfUfCfCmG-3'3'-dT  
(VEGF ligand)

5

41. A Lipid Construct comprising the Complex of claim 27.

42. The Lipid Construct of claim 41 wherein said Complex is



Ligand component =

5'-TsTsTsTs mGaUaC mGmGaU mAaCrG mGrAmG aUmGrG rAaCaC mGaUaC mAaCmG TsTsTsTsT-3'  
(VEGF ligand)

10

43. The Complex of claim 7 wherein said VEGF Nucleic Acid Ligand was identified from a Candidate Mixture of Nucleic Acids according to the method comprising:  
a) contacting the Candidate Mixture with VEGF, wherein the Nucleic Acids having an increased affinity to VEGF relative to the Candidate Mixture may be partitioned from the remainder of the Candidate Mixture;

15

b) partitioning the increased affinity Nucleic Acids from the remainder of the Candidate Mixture; and

c) amplifying the increased affinity Nucleic Acids to yield a mixture of Nucleic Acids enriched for Nucleic Acids having an increased affinity for VEGF; whereby  
5 Nucleic Acid Ligands of VEGF are produced.

44. The Complex of claim 43 wherein the method further comprises repeating steps b) and c).

10 45. A method for treating a VEGF mediated disease or medical condition comprising administering a pharmaceutically effective amount of a Complex comprised of a VEGF Nucleic Acid Ligand comprising 2'F-modified nucleotides and a Non-Immunogenic, High Molecular Weight Compound or a Lipophilic Compound.

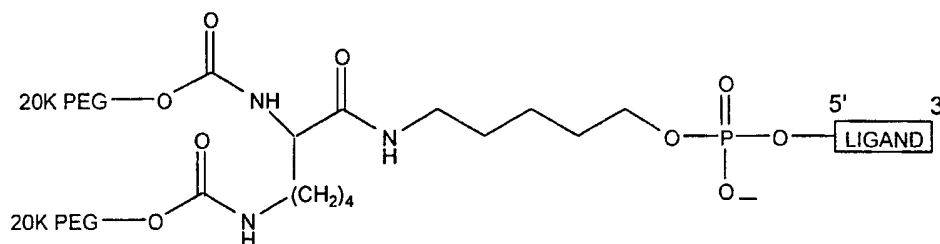
15 46. The method of claim 45 wherein said Non-Immunogenic, High Molecular Weight Compound is a Polyalkylene Glycol.

20 47. The method of claim 46 wherein said Polyalkylene Glycol is polyethylene glycol.

48. The method of claim 47 wherein said polyethylene glycol has a molecular weight of about between 10-80 K.

25 49. The method of claim 47 wherein said polyethylen glycol has a molecular weight of about 20-45 K.

50. The method of claim 49 wherein said Complex has the structure



Ligand Component =

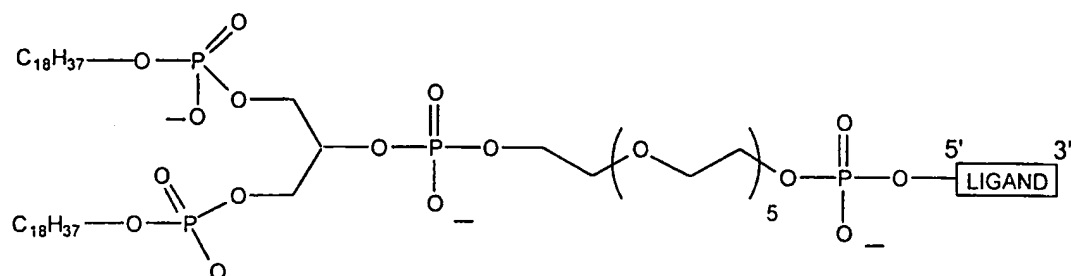
fCmGmGrArAfUfCmAmGfUmGmAmAfUmGfCfUfUmAfUmAfCmAfUfCfCmG-3'3'-dT  
(VEGF ligand)

30

51. The method of claim 45 wherein said Lipophilic Compound is a glycerol lipid.

52. The method of claim 51 wherein said Complex is further associated with a lipid construct.

53. The method of claim 52 wherein said Complex has the structure



Ligand Component =

fCmGmGrArAfUfCmAmGfUmGmAmAfUmGfCfUfUmAfUmAfCmAfUfCfCmG-3'3'-dT  
(VEGF ligand)

54. A method for the preparation of a Complex comprised of a VEGF Nucleic  
10 Acid Ligand comprising 2'-F-modified nucleotides and a Non-Immunogenic, High  
Molecular Weight Compound or Lipophilic Compound, said method comprising:

- a) identifying a VEGF Nucleic Acid Ligand from a Candidate Mixture of Nucleic Acids by the method comprising:
  - b) contacting the Candidate Mixture with VEGF, wherein Nucleic Acids  
15 having an increased affinity to VEGF relative to the Candidate Mixture may be partitioned from the remainder of the Candidate Mixture;
  - c) partitioning the increased affinity VEGF Nucleic Acids from the remainder of the Candidate Mixture;
  - e) amplifying the increased affinity VEGF Nucleic Acids to yield a ligand-  
20 enriched mixture of Nucleic Acids; and
  - f) associating said identified VEGF Nucleic Acid Ligand with a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound.

55. The method of claim 54 wherein said Complex is further associated with a  
25 Lipid Construct.

56. The method of claim 55 wherein said Lipid Construct is a Liposome.

57. The method of claim 56 wherein said Complex is comprised of a Nucleic  
30 Acid Ligand and a Lipophilic Compound and wherein said Complex is passively associated with the bilayer of said Liposomes by the method comprising the steps of:

- a) forming a liposome; and
- b) mixing said Complex comprised of a Nucleic Acid Ligand and a Lipophilic Compound with the Liposomes of step a) whereby the Nucleic Acid Ligand

Component of said Complex becomes associated with the bilayer of the Liposome and projects from the exterior of the Lipid bilayer.

5 58. The method of claim 54 wherein said Non-Immunogenic, High Molecular Weight Compound is Polyalkylene Glycol.

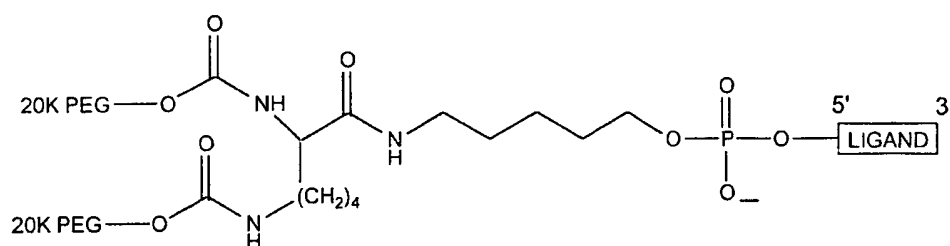
59. The method of claim 58 wherein said Polyalkylene Glycol is polyethylene glycol.

10 60. The method of claim 59 wherein said polyethylene glycol has a molecular weight of about between 10-80 K.

61. The method of claim 60 wherein said polyethylen glycol has a molecular weight of about 20-45 K.

15

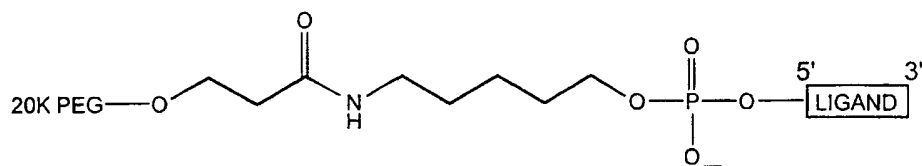
62. The method of claim 61 wherein said Complex has the structure



Ligand Component =

fCmGmGrArAfUfCmAmGfUmGmAmAfUmGfCfUfUmAfUmAfCmAfUfCfCmG-3'3'-dT  
(VEGF ligand)

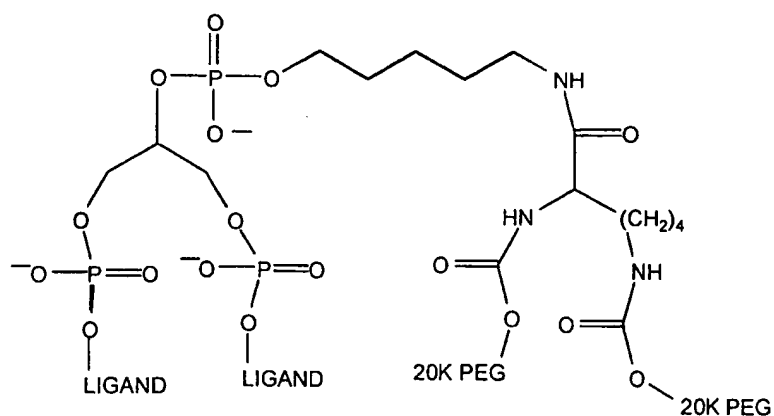
20 63. The method of claim 61 wherein said Complex has the structure



Ligand Component =

fCmGmGrArAfUfCmAmGfUmGmAmAfUmGfCfUfUmAfUmAfCmAfUfCfCmG-3'3'-dT  
(VEGF ligand)

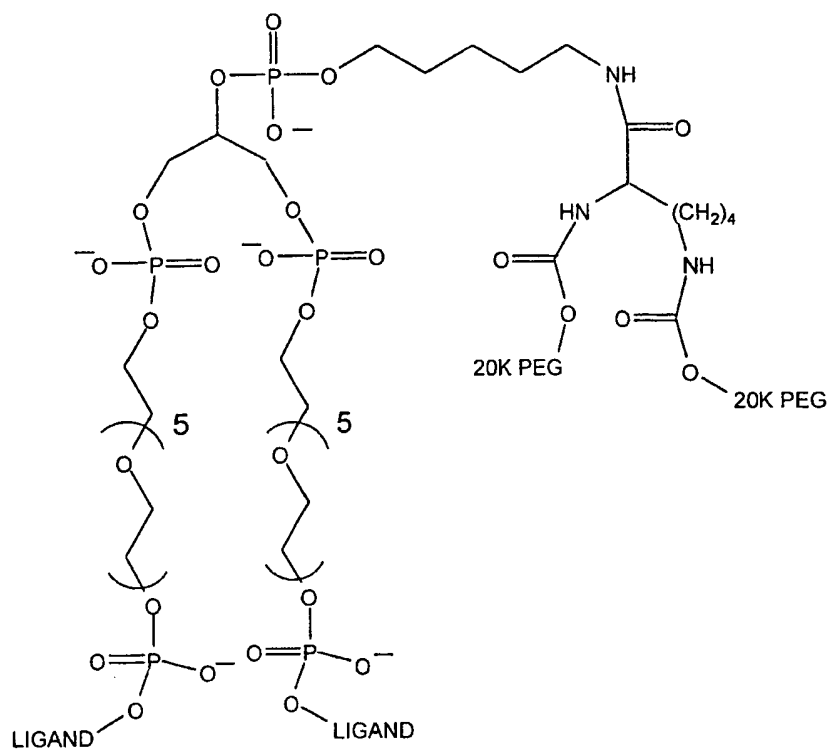
64. The method of claim 61 wherein said Complex has the structure



Ligand Component =

fCmGmGrArAfUfCmAmGfUmGmAmAfUmGfCfUfUmAfUmAfCmAfUfCfCmG-3'3'-dT  
(VEGF ligand)

65. The method of claim 61 wherein said Complex has the structure

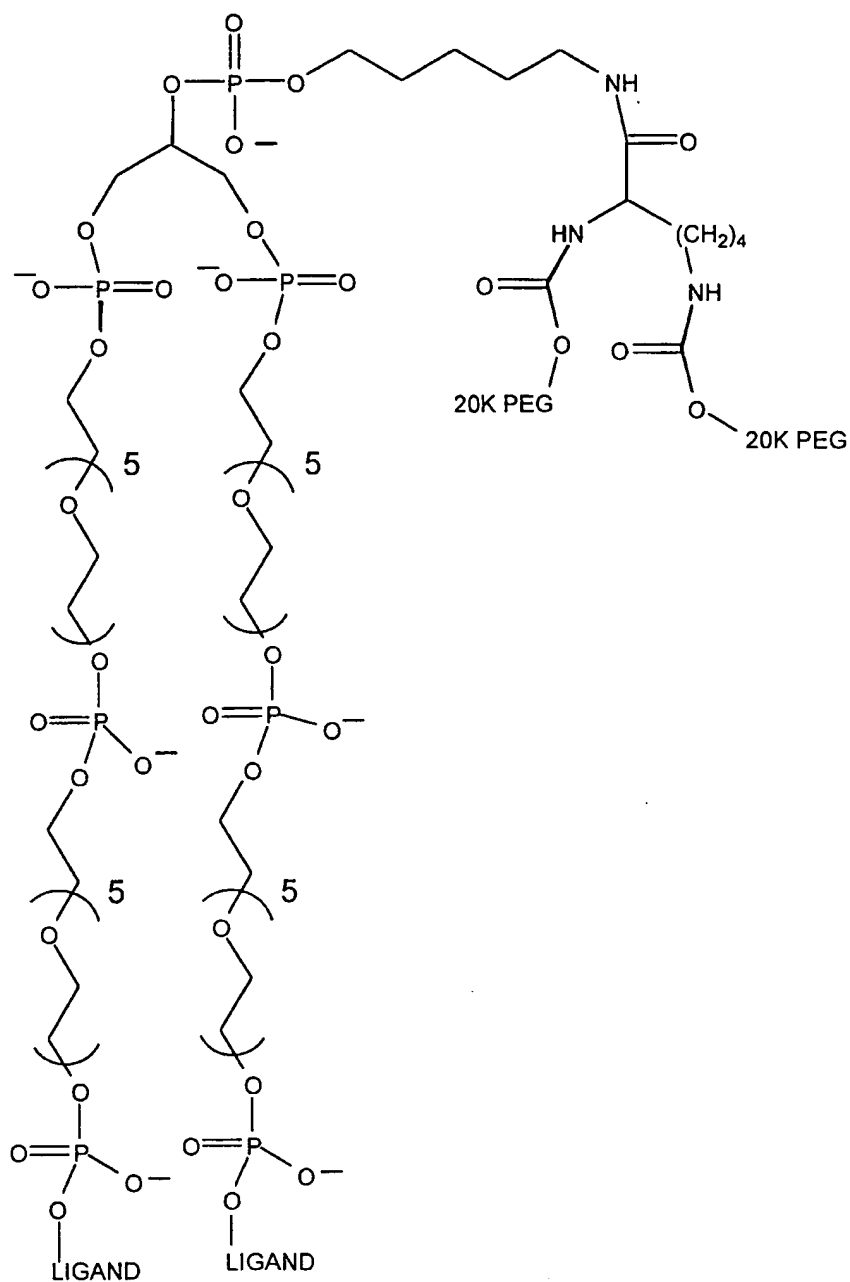


Ligand Component =

fCmGmGrArAfUfCmAmGfUmGmAmAfUmGfCfUfUmAfUmAfCmAfUfCfCmG-3'3'-dT  
(VEGF ligand)



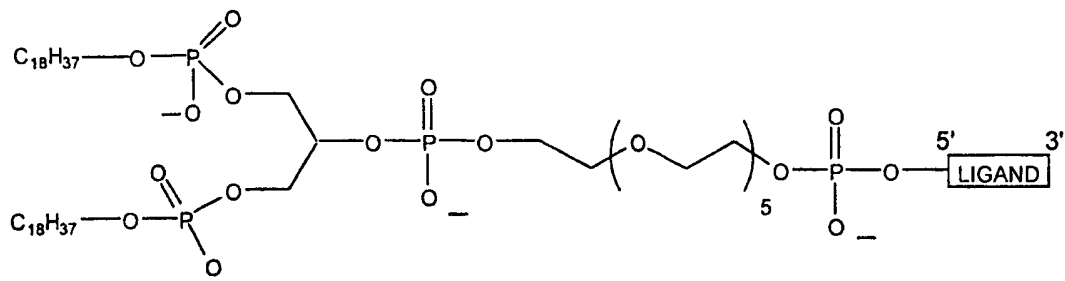
66. The method of claim 61 wherein said Complex has the structure



Ligand Component =

fCmGmGrArAfUfCmAmGfUmGmAmAfUmGfCfUfUmAfUmAfCmAfUfCfCmG-3'3'-dT  
(VEGF ligand)

67. The method of claim 55 wherein said Complex is

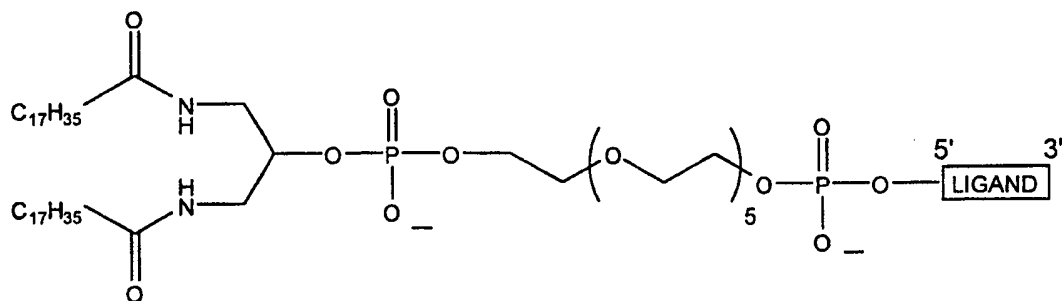


Ligand Component =

fCmGmGrArAfUfCmAmGfUmGmAmAfUmGfCfUfUmAfUmAfCmAfUfCfCmG-3'3'-dT  
(VEGF ligand)

5

68. The method of claim 55 wherein said Complex is

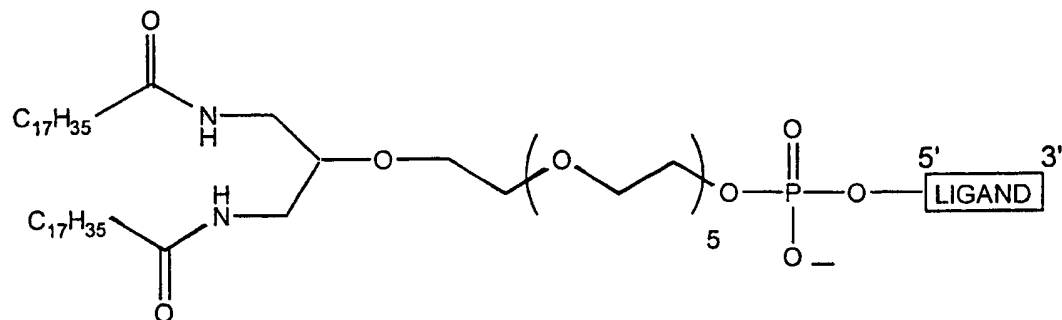


Ligand Component =

fCmGmGrArAfUfCmAmGfUmGmAmAfUmGfCfUfUmAfUmAfCmAfUfCfCmG-3'3'-dT  
(VEGF ligand)

10

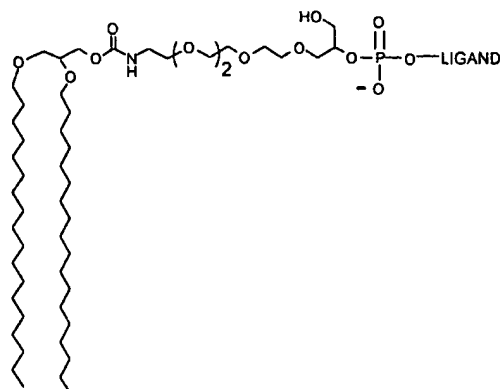
69. The method of claim 55 wherein said Complex is



Ligand Component =

fCmGmGrArAfUfCmAmGfUmGmAmAfUmGfCfUfUmAfUmAfCmAfUfCfCmG-3'3'-dT  
(VEGF ligand)

70. The method of claim 55 wherein said Complex is



Ligand component =

5'-TsTsTsTs mGaUaC mGmGaU mAaCrG mGrAmG aUmGrG rAaCaC mGaUaC mAaCmG TsTsTsTsT-3'  
(VEGF ligand)

5

71. A method for improving the pharmacokinetic properties of a VEGF Nucleic Acid Ligand comprising 2'F-modified nucleotides comprising:  
covalently linking said VEGF Nucleic Acid Ligand with a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound to form a  
10 Complex comprised of a VEGF Nucleic Acid Ligand and a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound; and administering said Complex to a patient.

72. A method for targeting a therapeutic or diagnostic agent to a specific  
15 predetermined biological target that is expressing VEGF in a patient comprising:  
covalently linking said therapeutic or diagnostic agent with a Complex comprised of a VEGF Nucleic Acid Ligand comprising 2'F-modified nucleotides and a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound, and administering said Complex to a patient.

20

73. A method of inhibiting VEGF mediated angiogenesis comprising:  
covalently linking a VEGF Nucleic Acid Ligand comprising 2'F-modified  
nucleotides to a Non-Immunogenic, High Molecular Weight Compound or Lipophilic  
Compound to form a Complex; and administering said Complex to a patient.

25

74. A method of inhibiting the growth of tumors comprising:  
covalently linking a VEGF Nucleic Acid Ligand comprising 2'F-modified  
nucleotides to a Non-Immunogenic, High Molecular Weight Compound or Lipophilic  
Compound to form a Complex; and administering said Complex to a patient.

30

75. The method of claim 74 wherein said tumors or cells or tissues surrounding said tumors are expressing VEGF.

76. The method of claim 74 wherein said tumors or cells or tissues surrounding said tumors are expressing VEGF receptors.

77. The method of claim 76 wherein said tumors are comprised of Kaposi's sarcoma cells.

78. A method of inhibiting macular degeneration comprising: covalently linking a VEGF Nucleic Acid Ligand comprising 2'F-modified nucleotides to a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound to form a Complex; and administering said Complex to a patient.

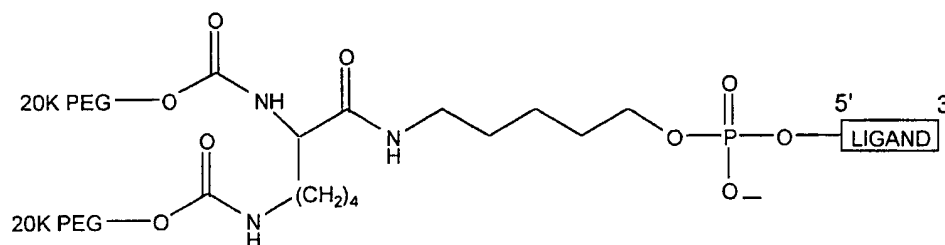
79. The method of claim 78 wherein said Non-Immunogenic, High Molecular Weight Compound is a polyalkylene glycol.

80. The method of claim 79 wherein said polyalkylene glycol is polyethylene glycol.

81. The method of claim 80 wherein said polyethylene glycol has a molecular weight of about between 10-80k.

82. The method of claim 80 wherein said polyethylene glycol has a molecular weight of about between 20-45k.

83. The method of claim 82 wherein said complex has the structure



Ligand Component =

fCmGmGrArAfUfCmAmGfUmGmAmAfUmGfCfUfUmAfUmAfCmAfUfCfCmG-3'3'-dT  
(VEGF ligand)

84. A method for prolonging the residence time of a Nucleic Acid Ligand in an ocular application comprising attaching a Non-Immunogenic, High Molecular Weight Compound to a Nucleic Acid Ligand to form a Complex, and administering said complex to the eye.

NX-213  
SEQ ID NO: 1

Ligand =

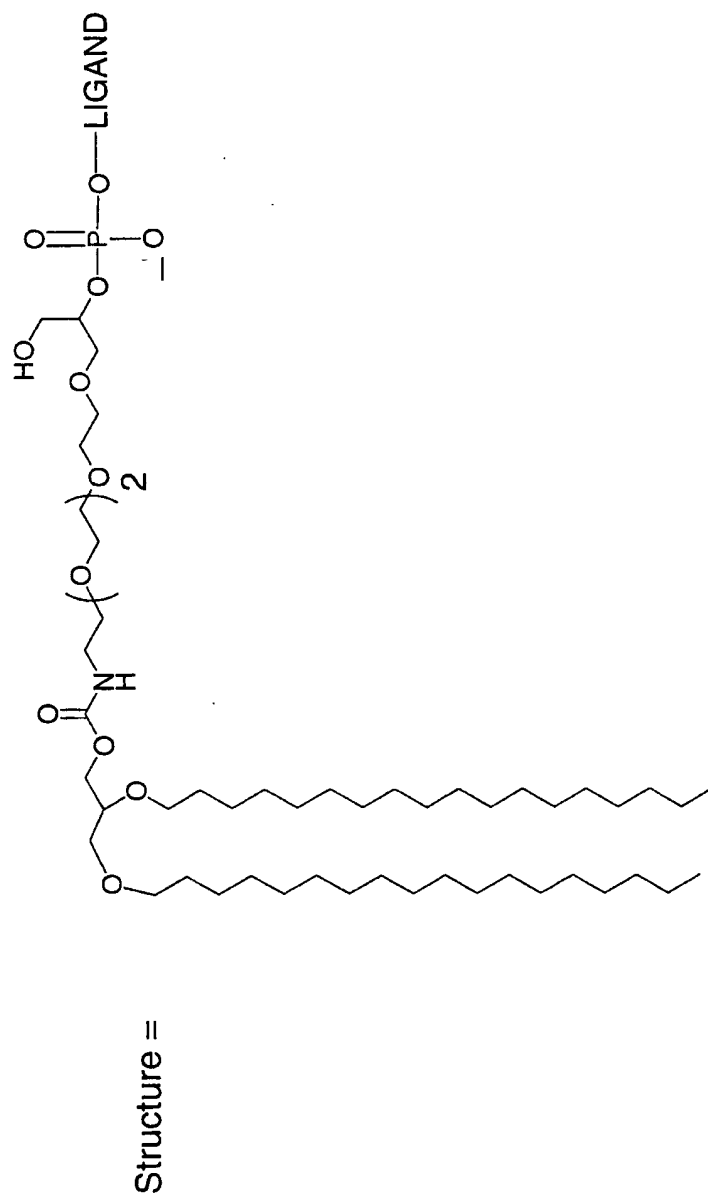
5'-TsTsTsmAaCaCaCaUrGrAaUmGrGaUmAmGrAaCmGaCaCmGmGmGaUmGTsTsTsT-3'

1/34

FIG. 1A

NX-278

SEQ ID NO: 2



Ligand component =

5'-TsTsTsTs mGaUaC mGmGaU mAaCrG mGrAmG aUmGrG rAaCaC mGaUaC mAaCmG TsTsTsTsT-3'  
(VEGF ligand)

FIG. 1B



scNX-213  
SEQ ID NO: 4

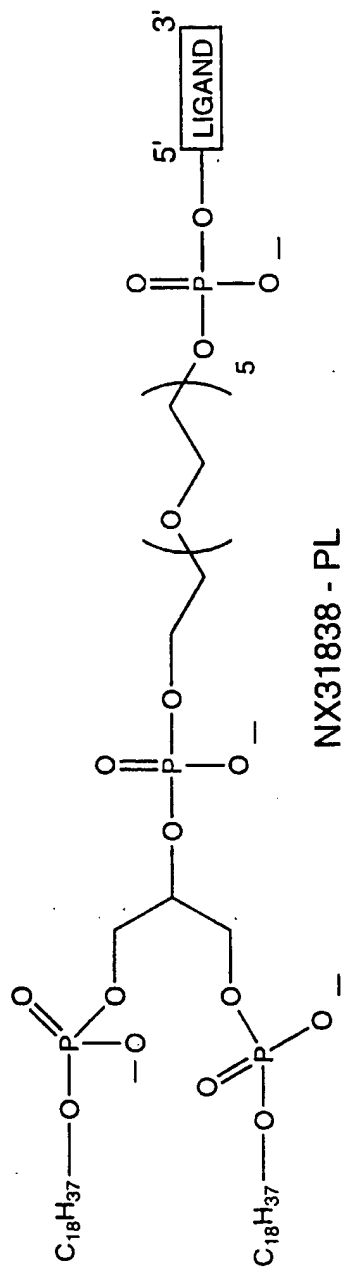
Ligand =

5'-TsTsTsTsmGaUaCmGmGaUmAaCrGmGrAmGaUmGrGrAaCaCmGaUaCmAaCmGTsTsTsT-3'

4/34

FIG. 1D



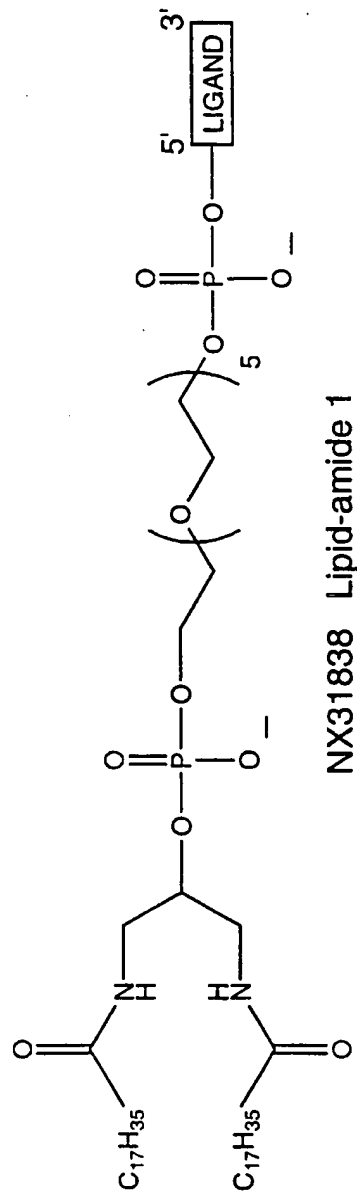


Ligand Component =

fCmGmGrArAfUfCmAfUmGmAfUmGfCfUfUmAfUmAfCmAfUfCfCmG-3'3'-dT  
(VEGF ligand)

FIG. 1E

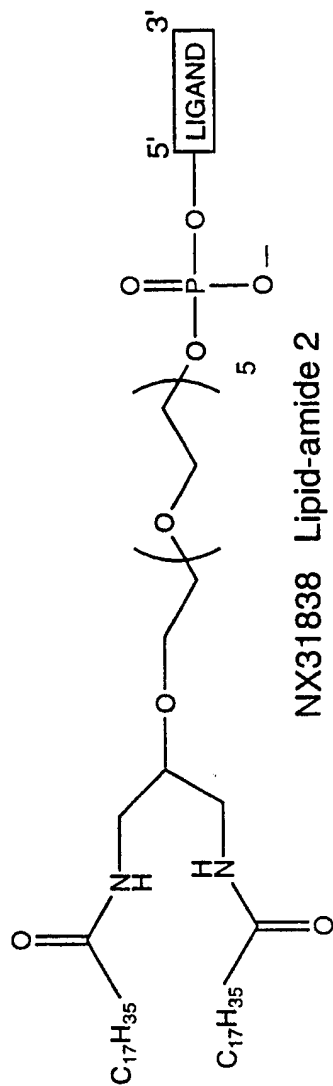
5/34



Ligand Component =

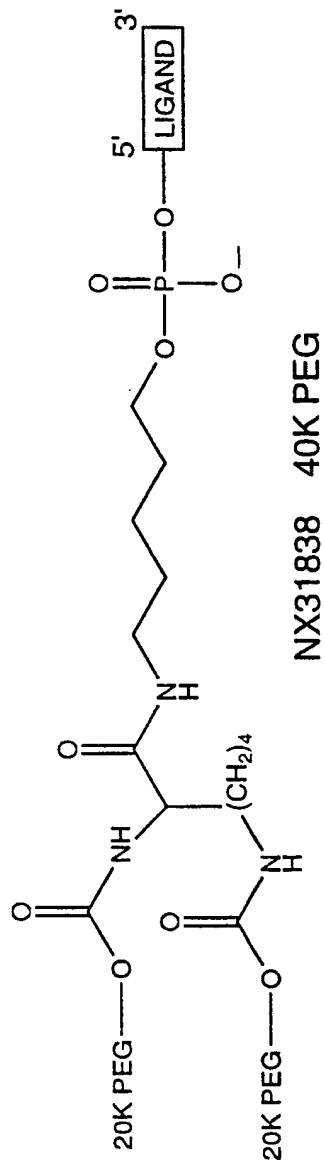
fCmGmGrArAfUfCmAfUmGmAfUmGfCfUfUmAfUmAfCmAfUfCfCmG-3'3'-dT  
(VEGF ligand)

FIG. 1F



Ligand Component =  
fCmGmGrArAfUfCmAmGfUmGmAmAfUmGfCfUfUmAfUmAfCmAfUfCfCmG-3'3'-dT  
(VEGF ligand)

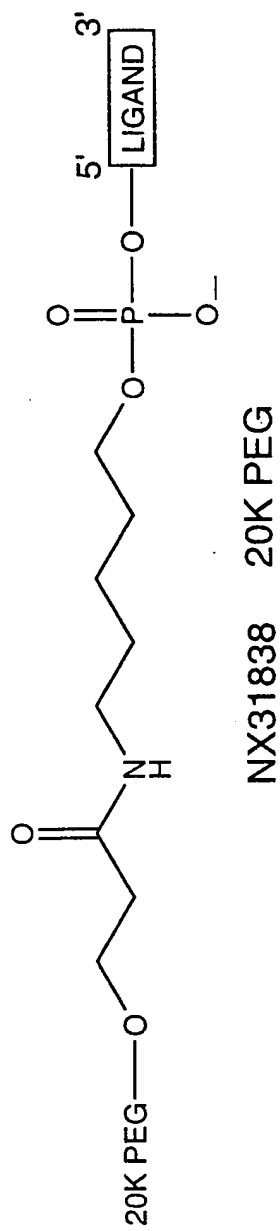
FIG. 1G



Ligand Component =  
fCmGmGrArAfUfCmAmGfUmGmAmAfUmGfCfUfUmAfUmAfCmAfUfCfCmG-3'3'-dT  
(VEGF ligand)

FIG. 1H

6/34

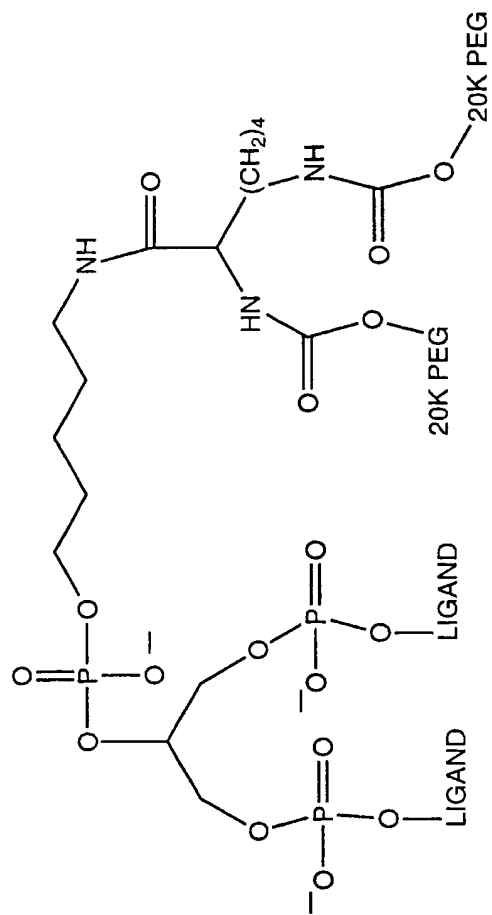


Ligand Component =

fCmGmGrArAfUfCmAmGfUmGmAmAfUmGfCfUfUmAfUmAfCmAfUfCfCmG-3'3'-dT  
(VEGF ligand)

FIG. 1I

7/34

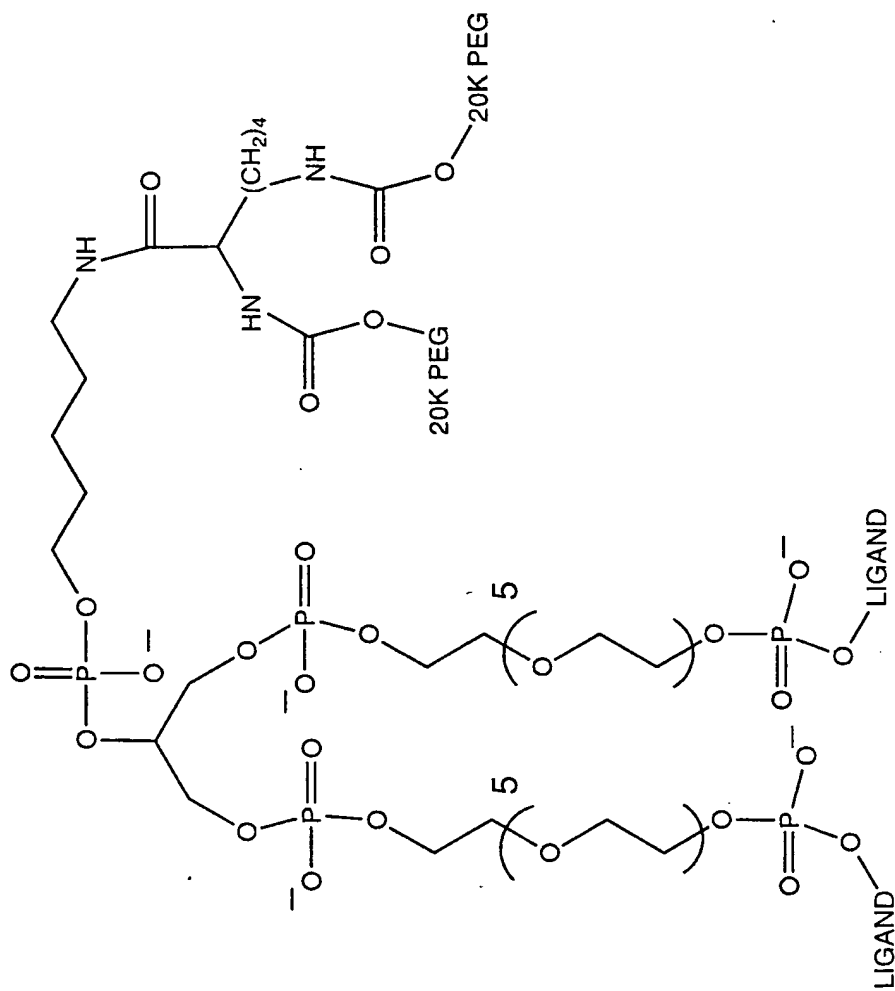


Ligand Component =

fCmGmGrArAfUfCmAmGfUmGmAmAfUmGfCfUfUmAfUmAfCmAfUfCfCmG-3'3'-dT  
 -(VEGF ligand)

8/34

FIG. 1J



Ligand Component =

fCmGmGrArAfUfCmAmGfUmGmAmAfUmGfCfUfUmAfUmAfCmAfUfCfCmG-3'3'-dT  
(VEGF ligand)

FIG. 1K

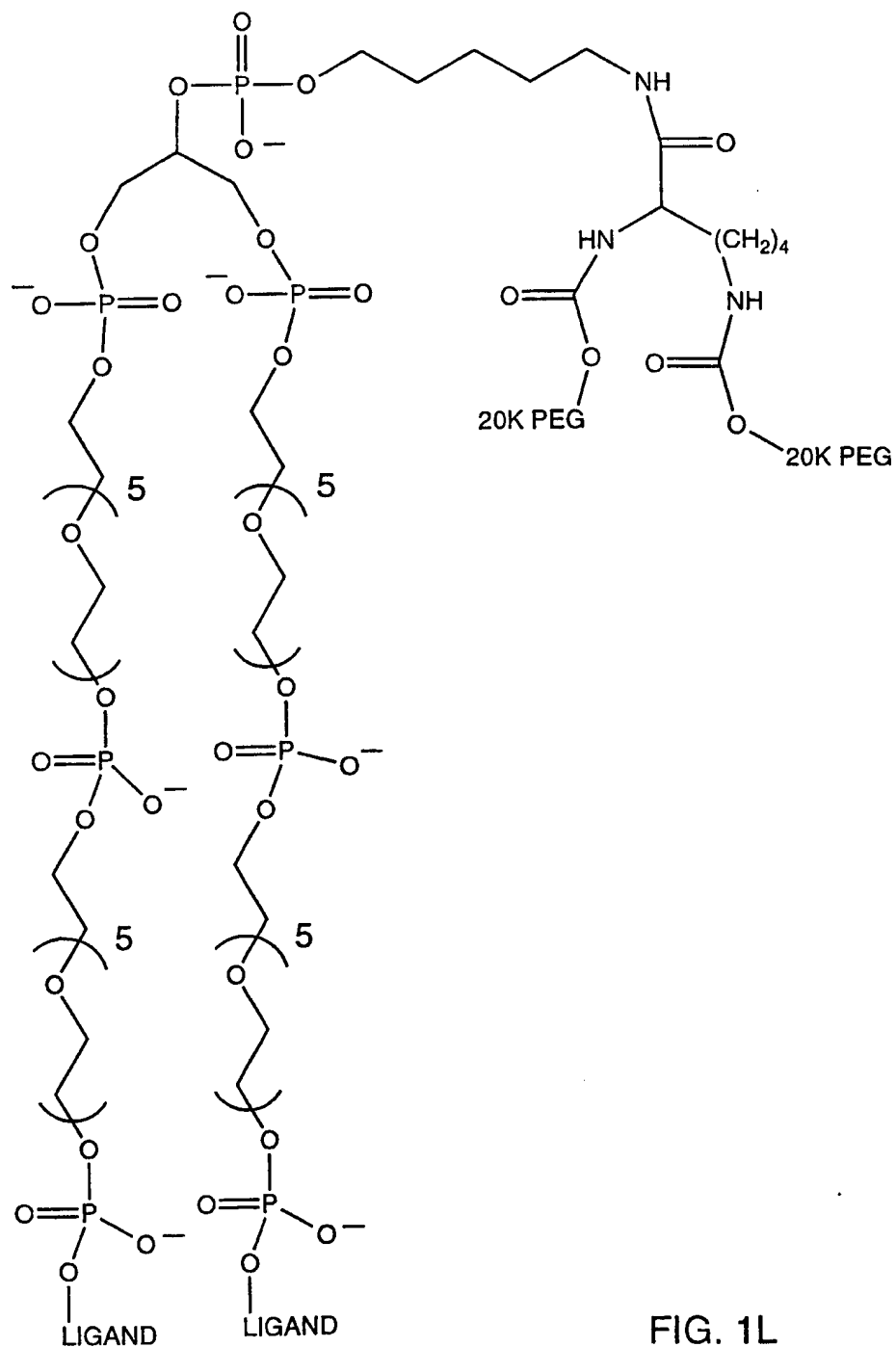
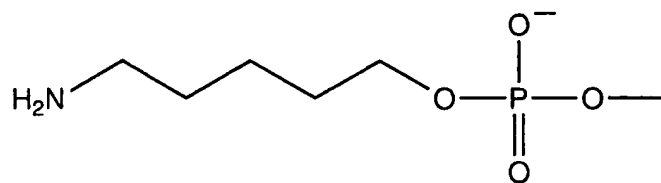


FIG. 1L

Ligand Component =

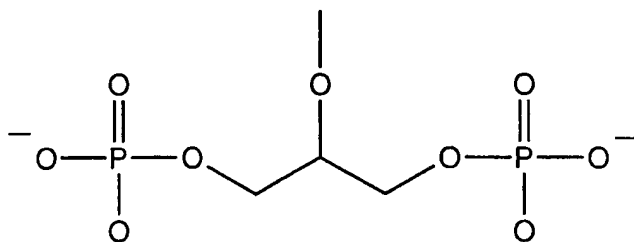
fCmGmGrArAfUfCmAmGfUmGmAmAfUmGfCfUfUmAfUmAfCmAfUfCfCmG-3'3'-dT  
(VEGF ligand)

10/34



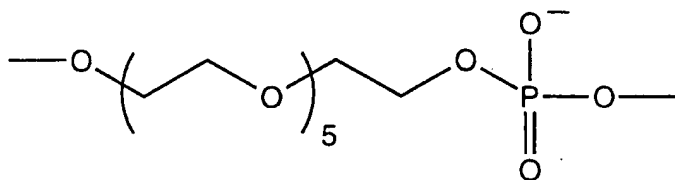
C-5 Amino linker

FIG. 1M



Glycerol bisphosphate

FIG. 1N



18 atom spacer

FIG. 1O

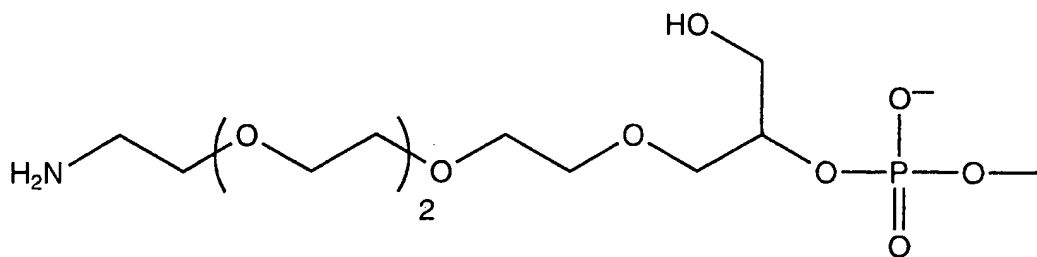


FIG. 1P

11/34

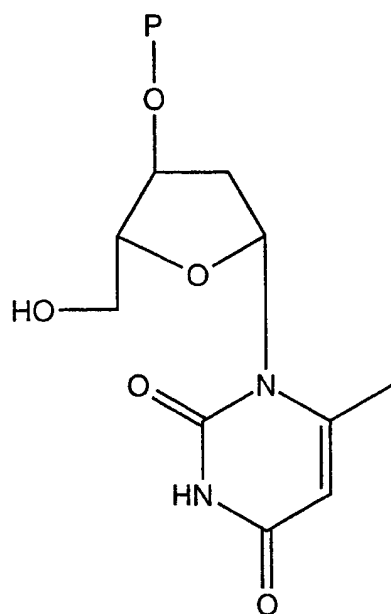
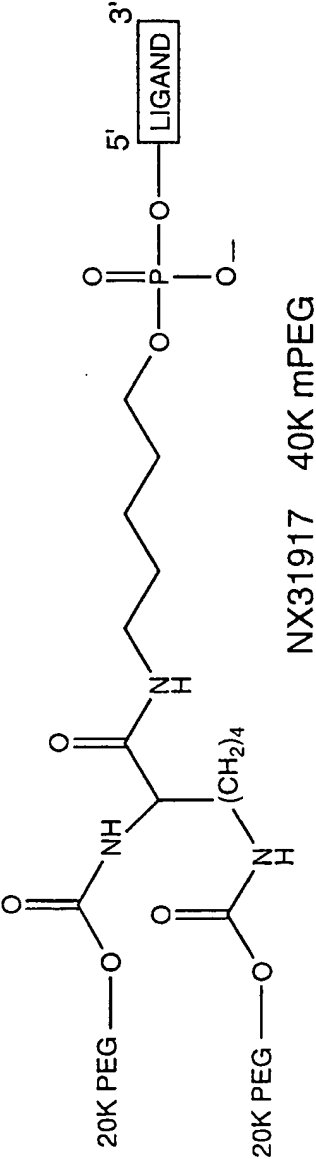


FIG. 1Q

12/34





Ligand Component =

5'-[C5 NH<sub>2</sub> link]-fCrAfUfUmAfCmAfCfCmGrAmGfUfUfUmAfCmGfUmGmAmGfUmAfUmG-3'3'dT-3'

13/34

FIG. 1R

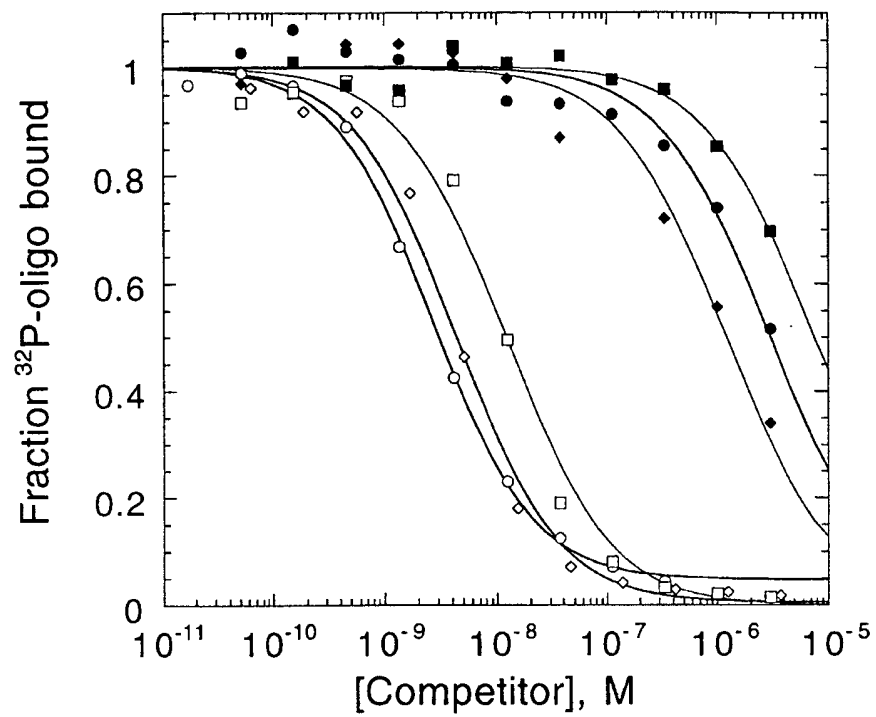


FIG. 2

14/34

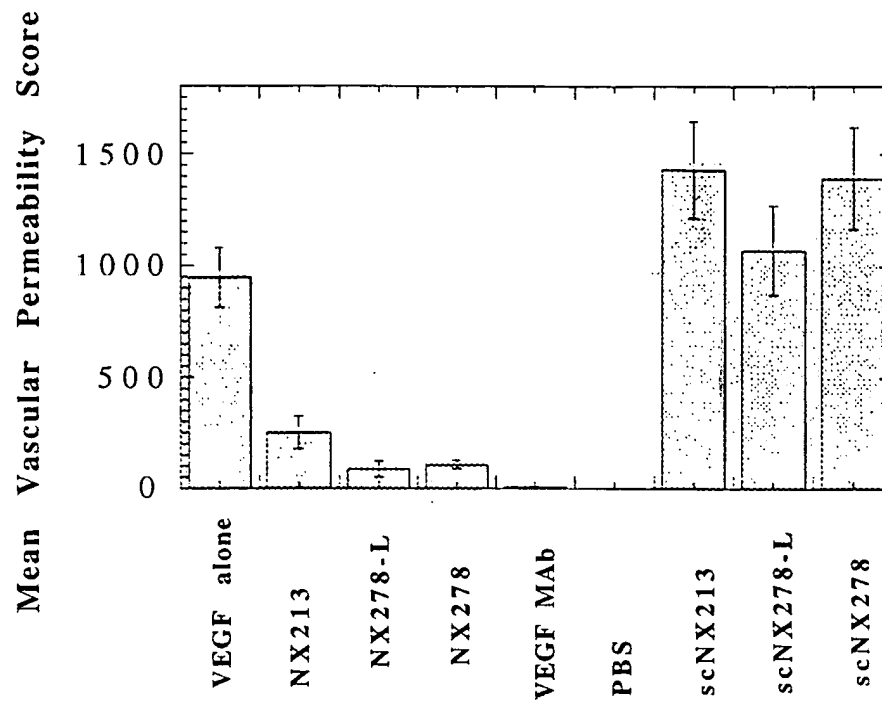


FIG. 3

15/34

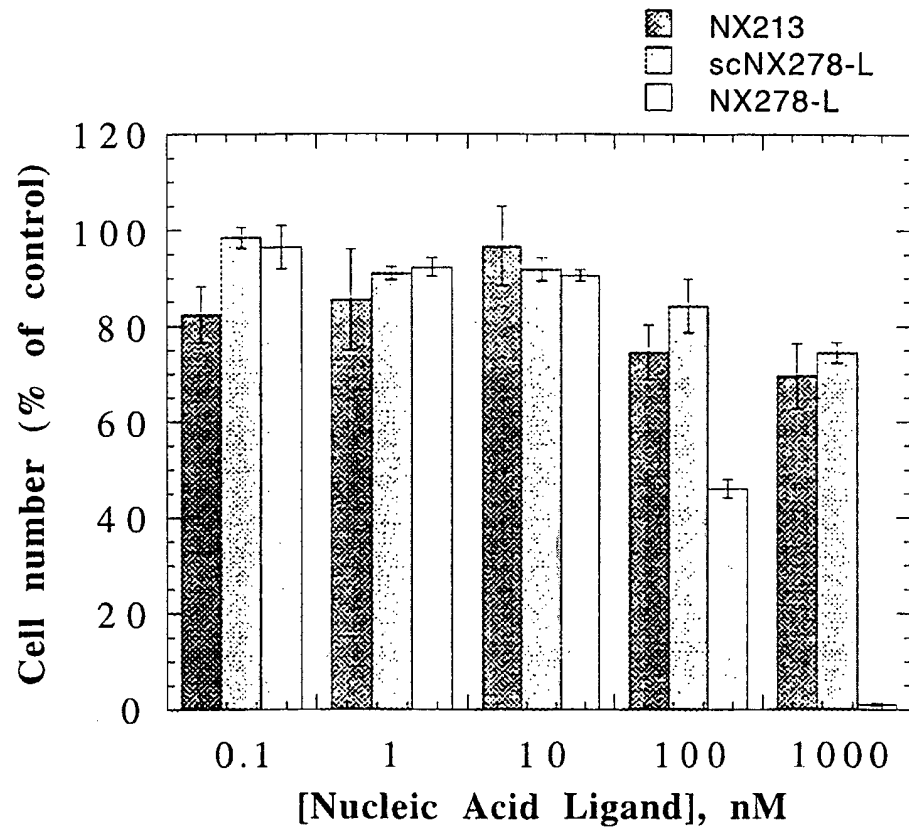


FIG. 4

16/34

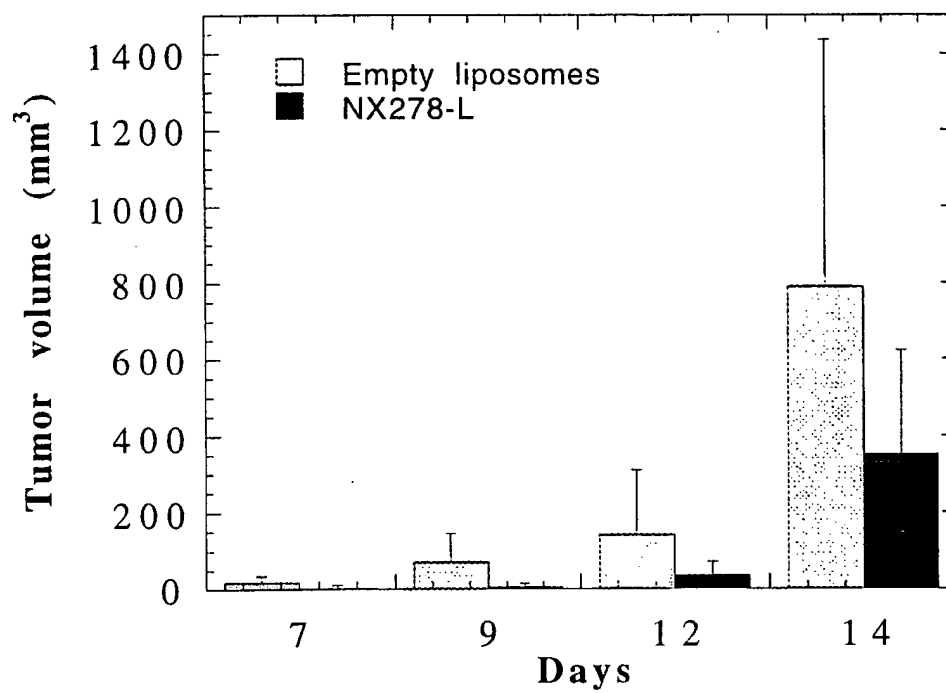


FIG. 5A

17/34

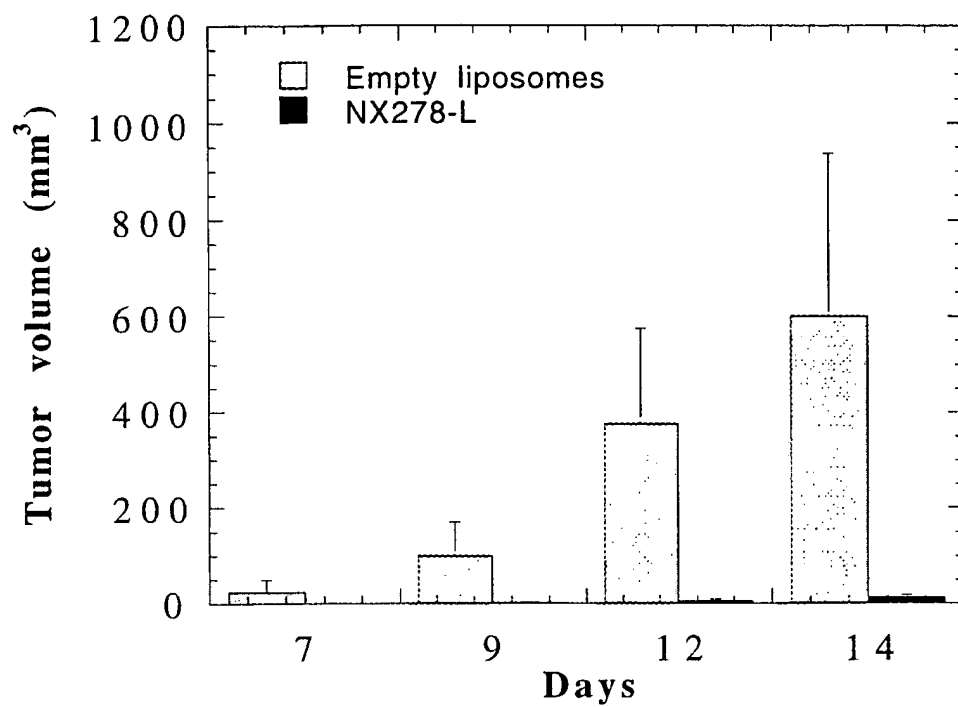
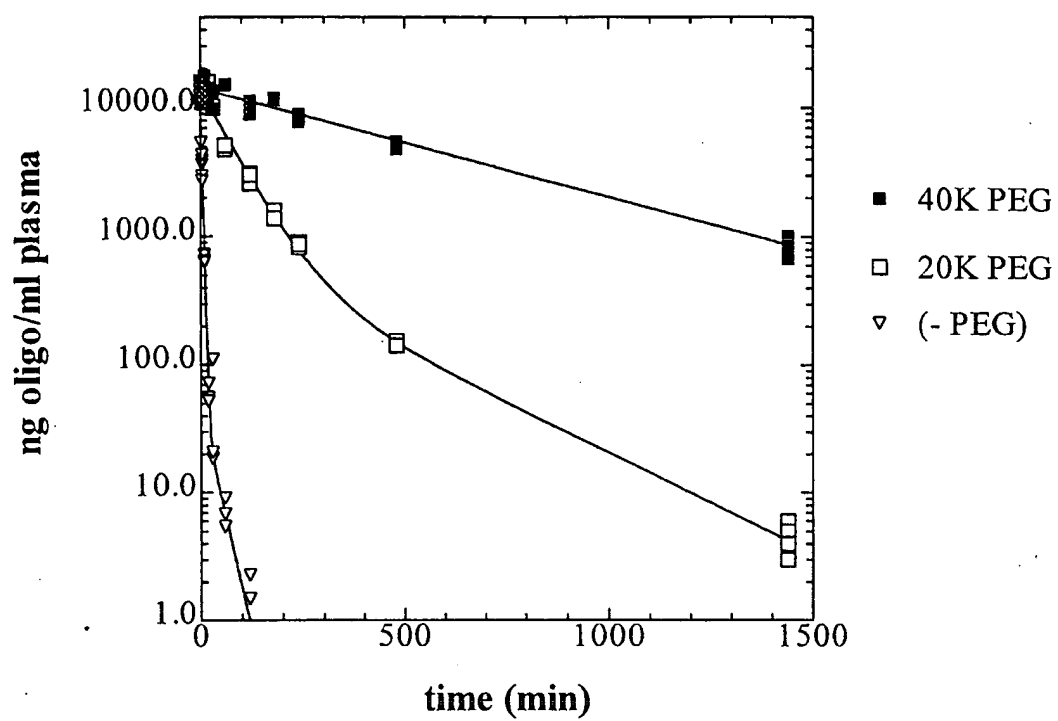


FIG. 5B

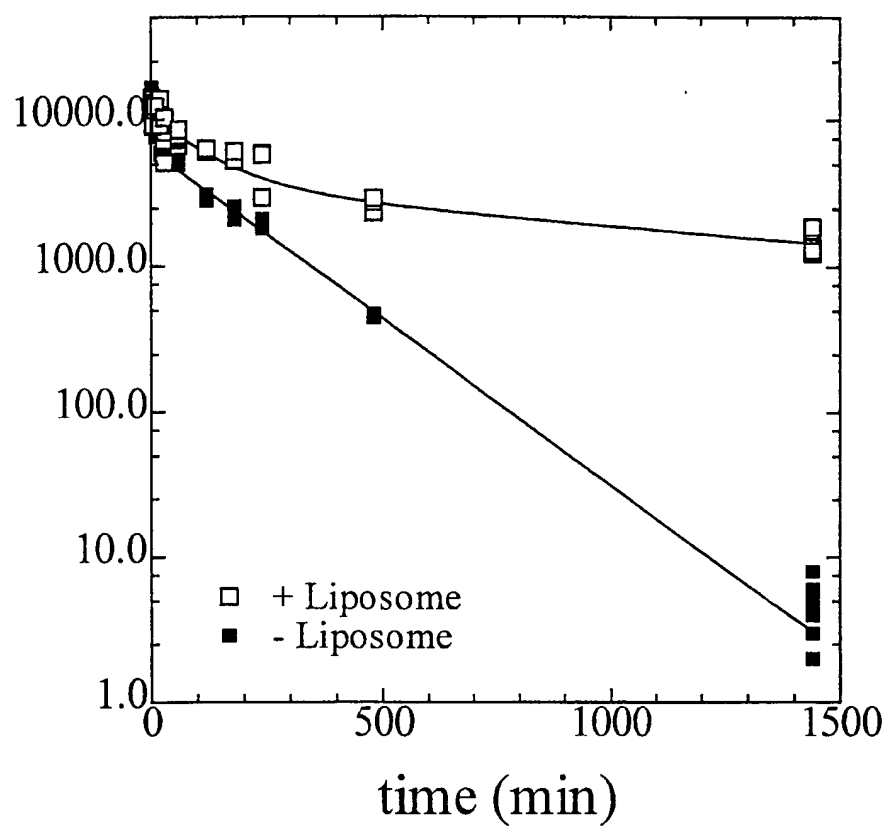
10/34

Figure 6



19/34

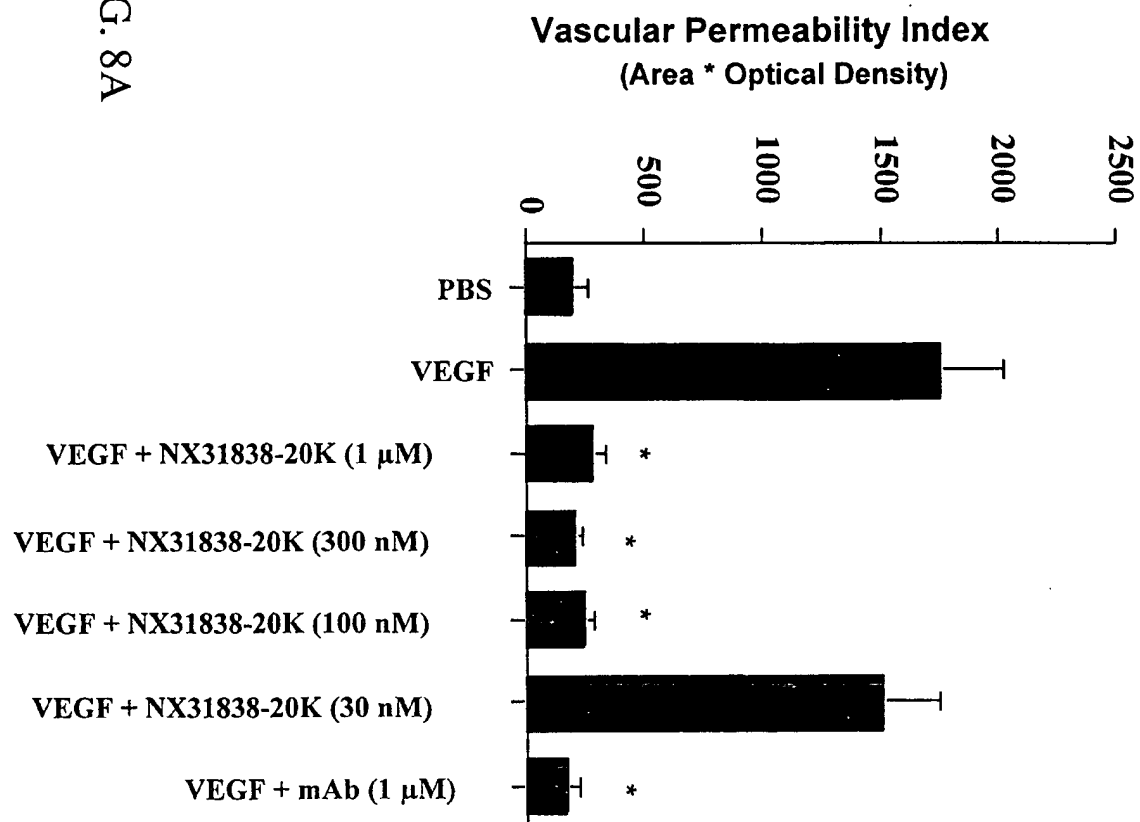
Figure 7



20/34



FIG. 8A



21/34

FIG. 8B

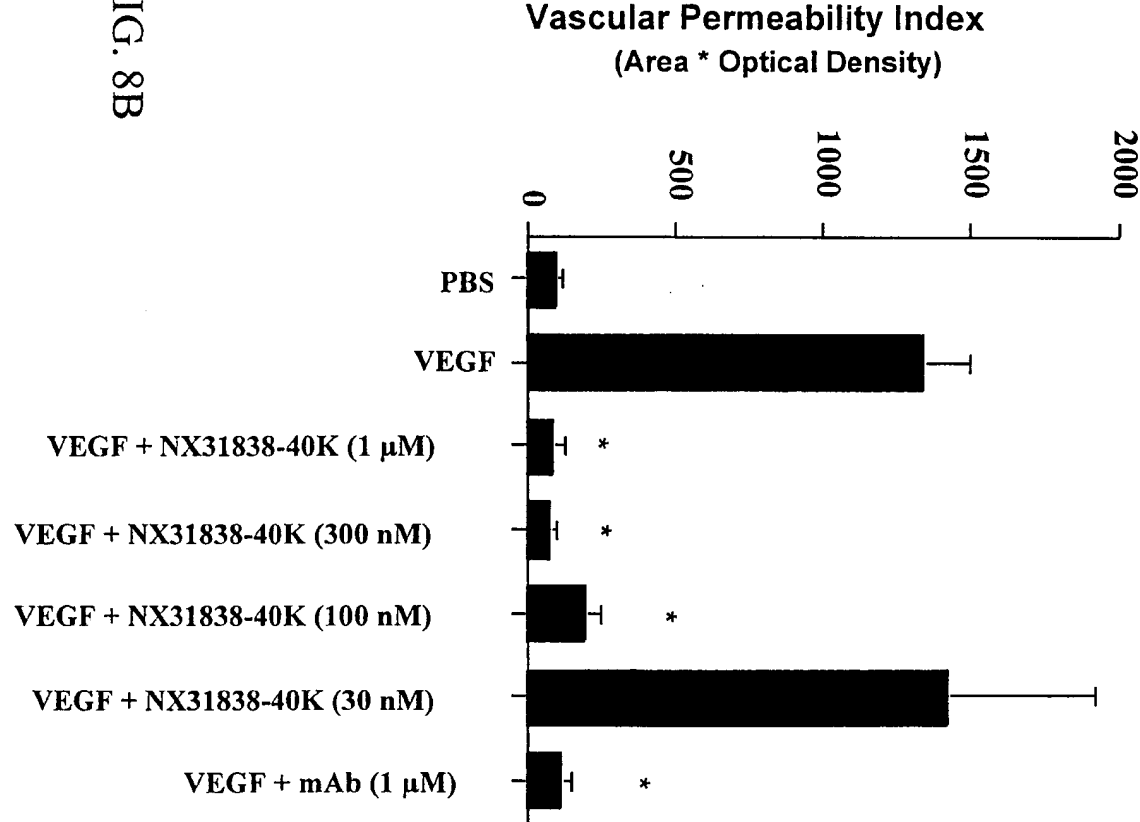
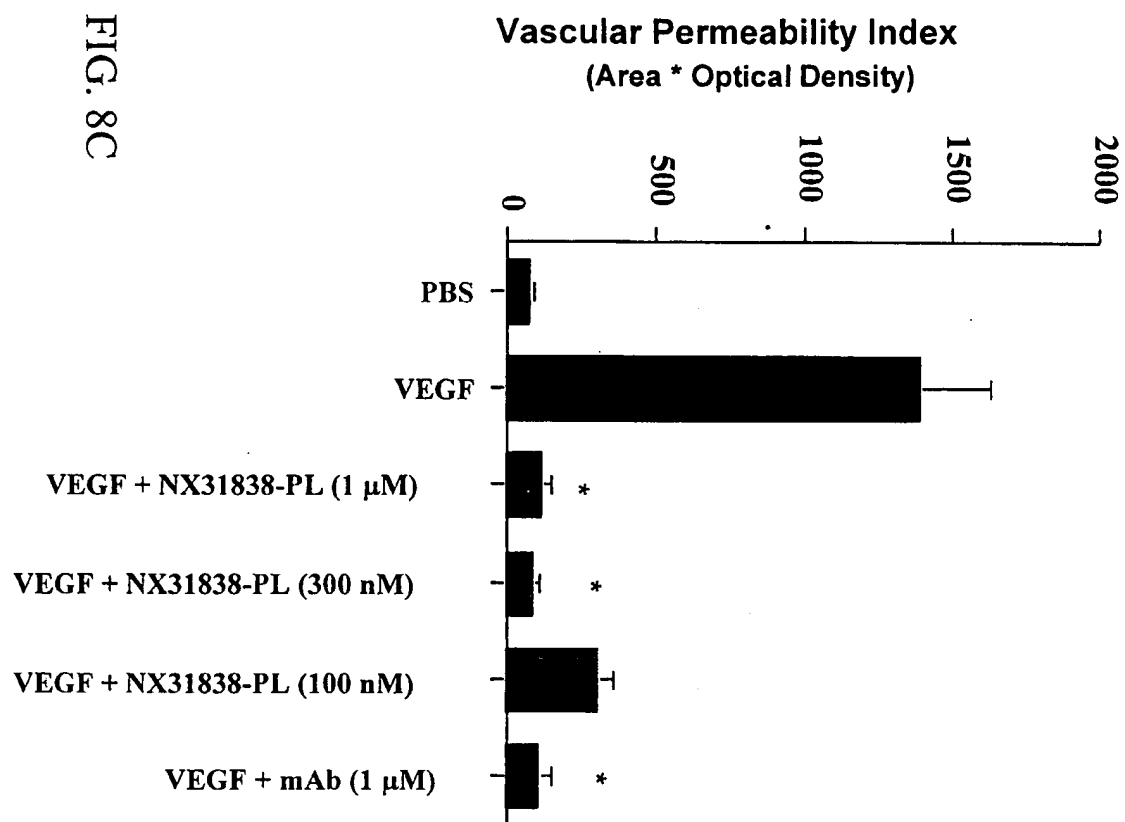
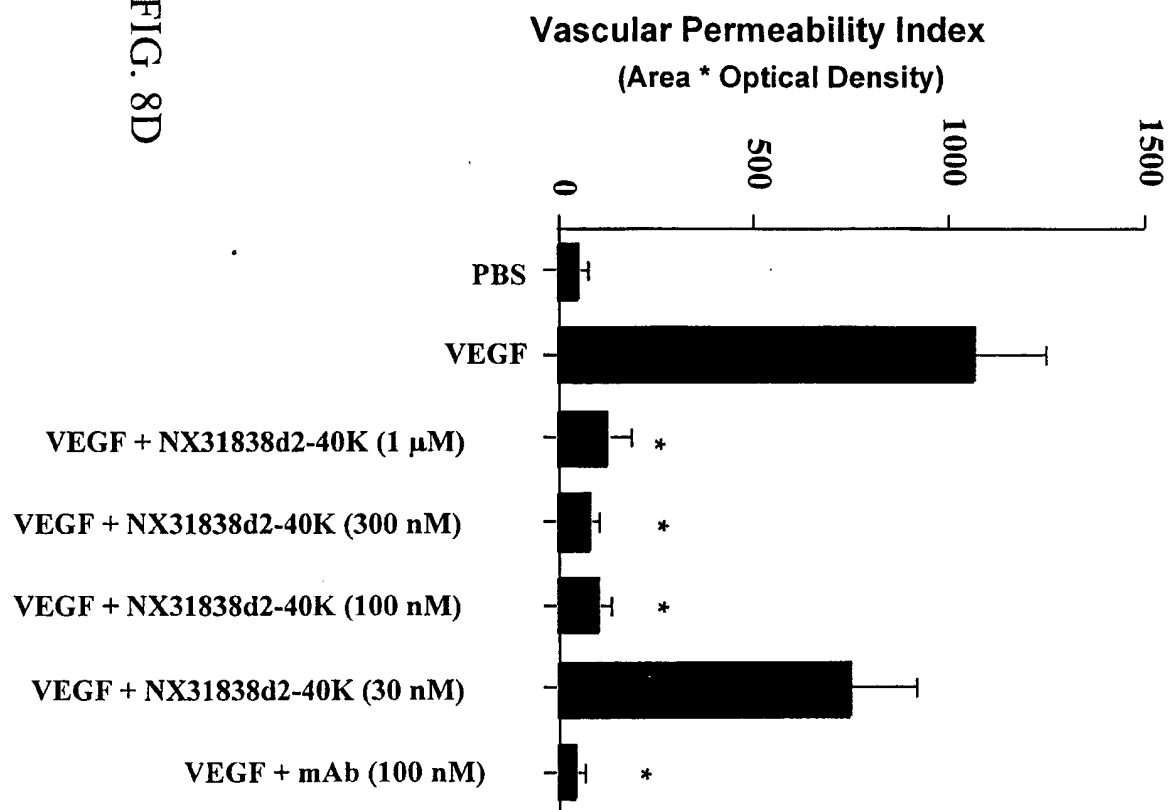


FIG. 8C



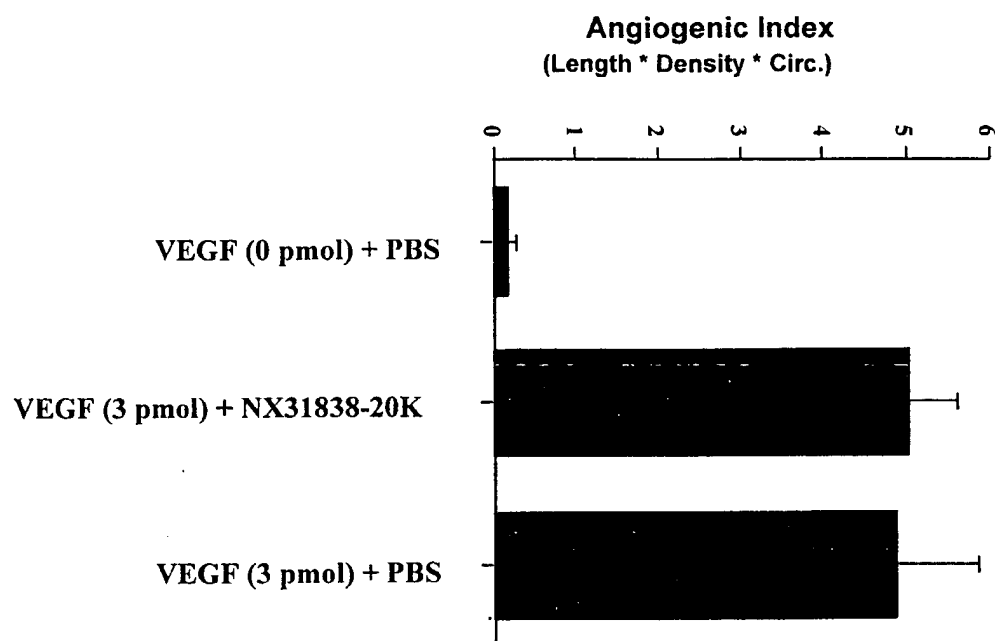
73/34

FIG. 8D



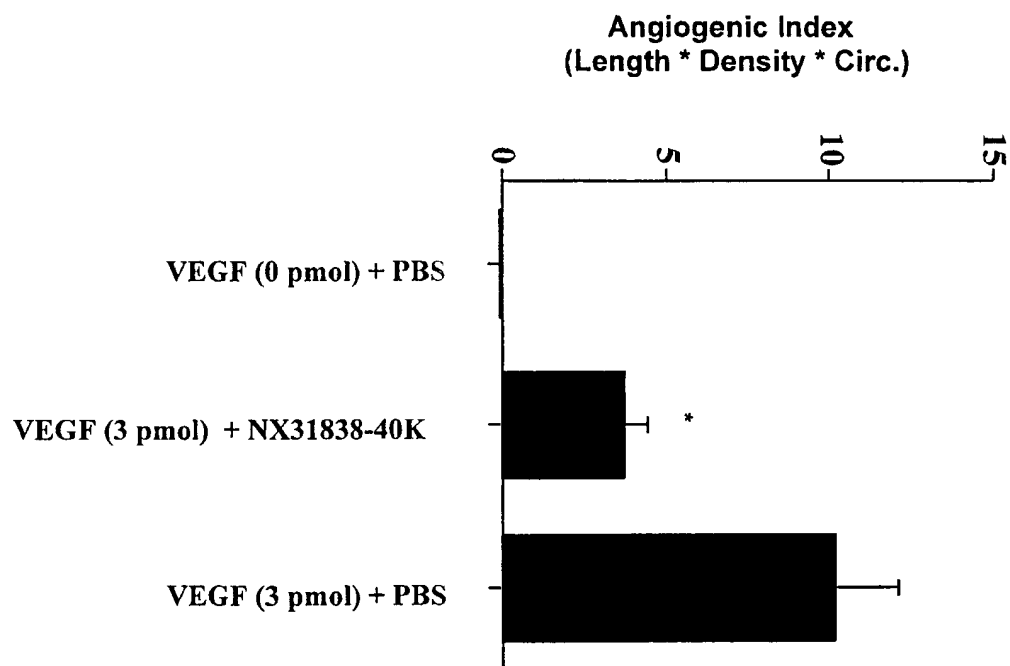
24/34

FIG. 9A



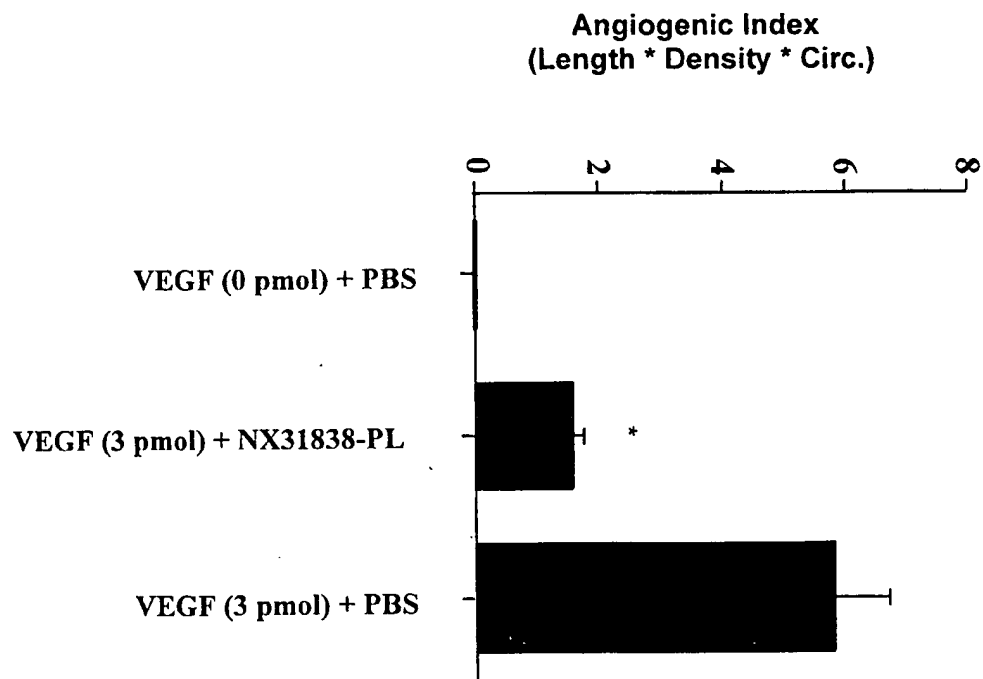
25/34

FIG. 9B



26/34

FIG. 9C



22/34

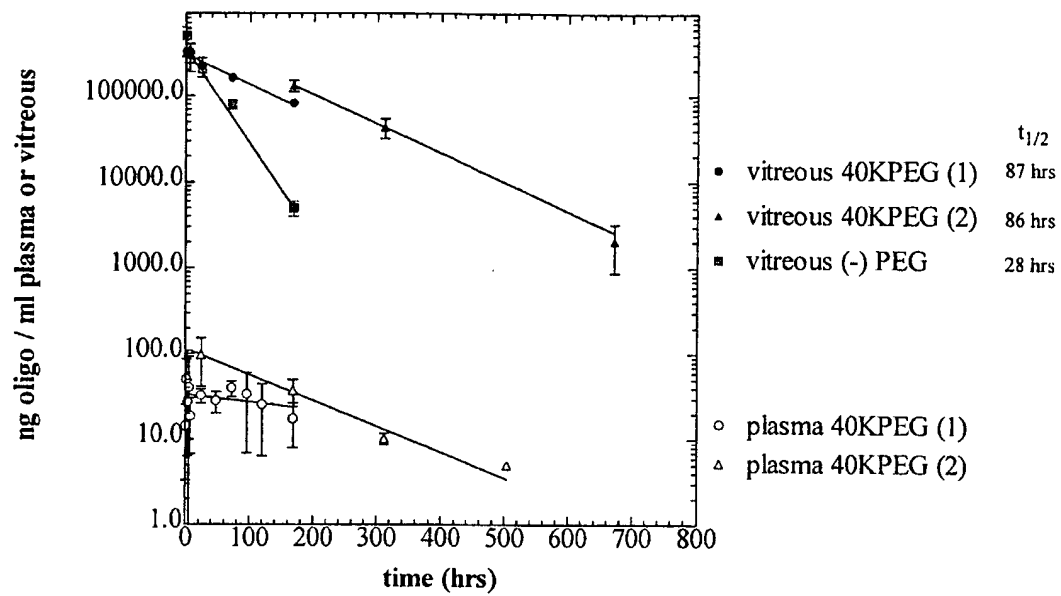


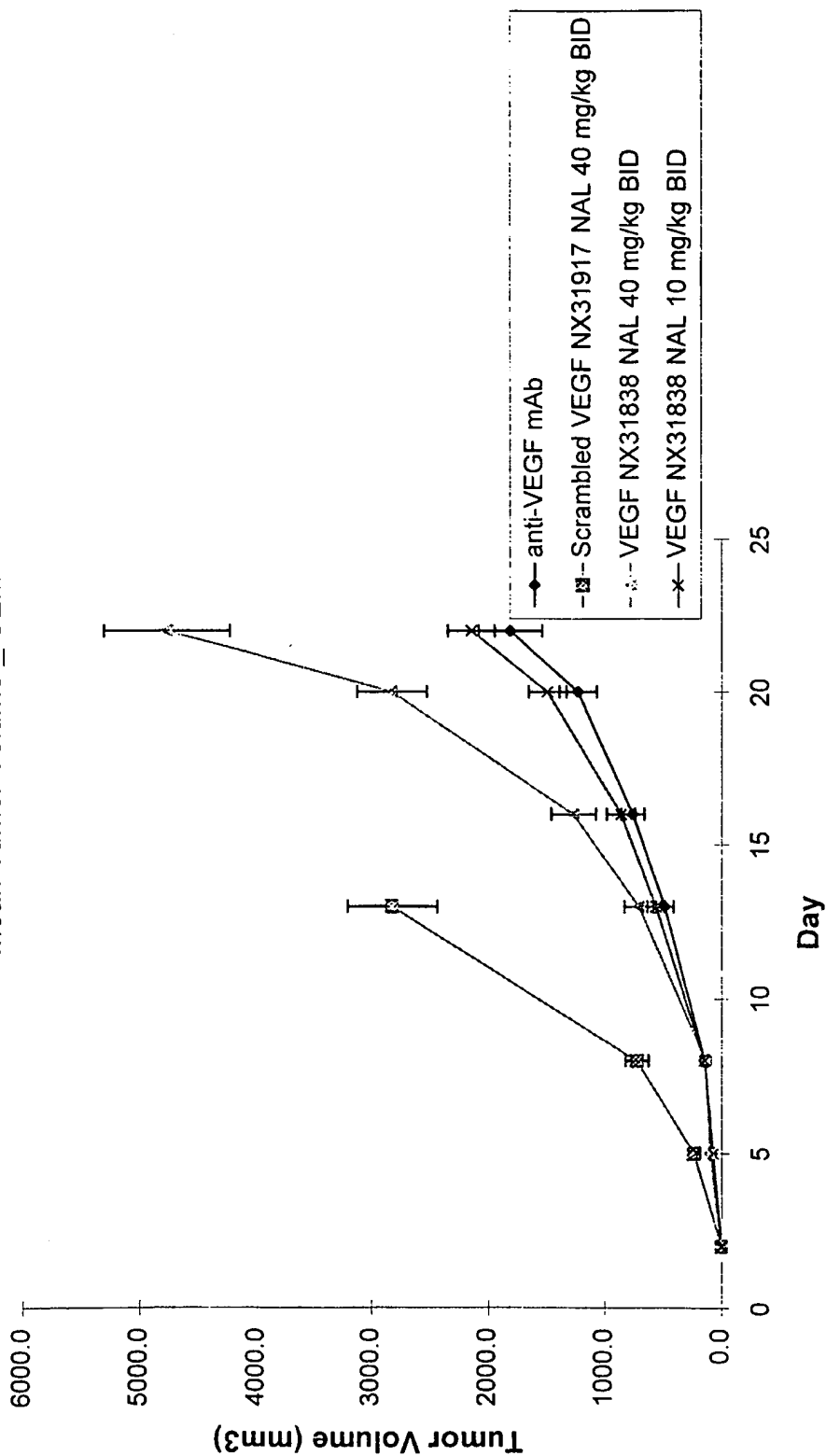
FIG. 10

29/34



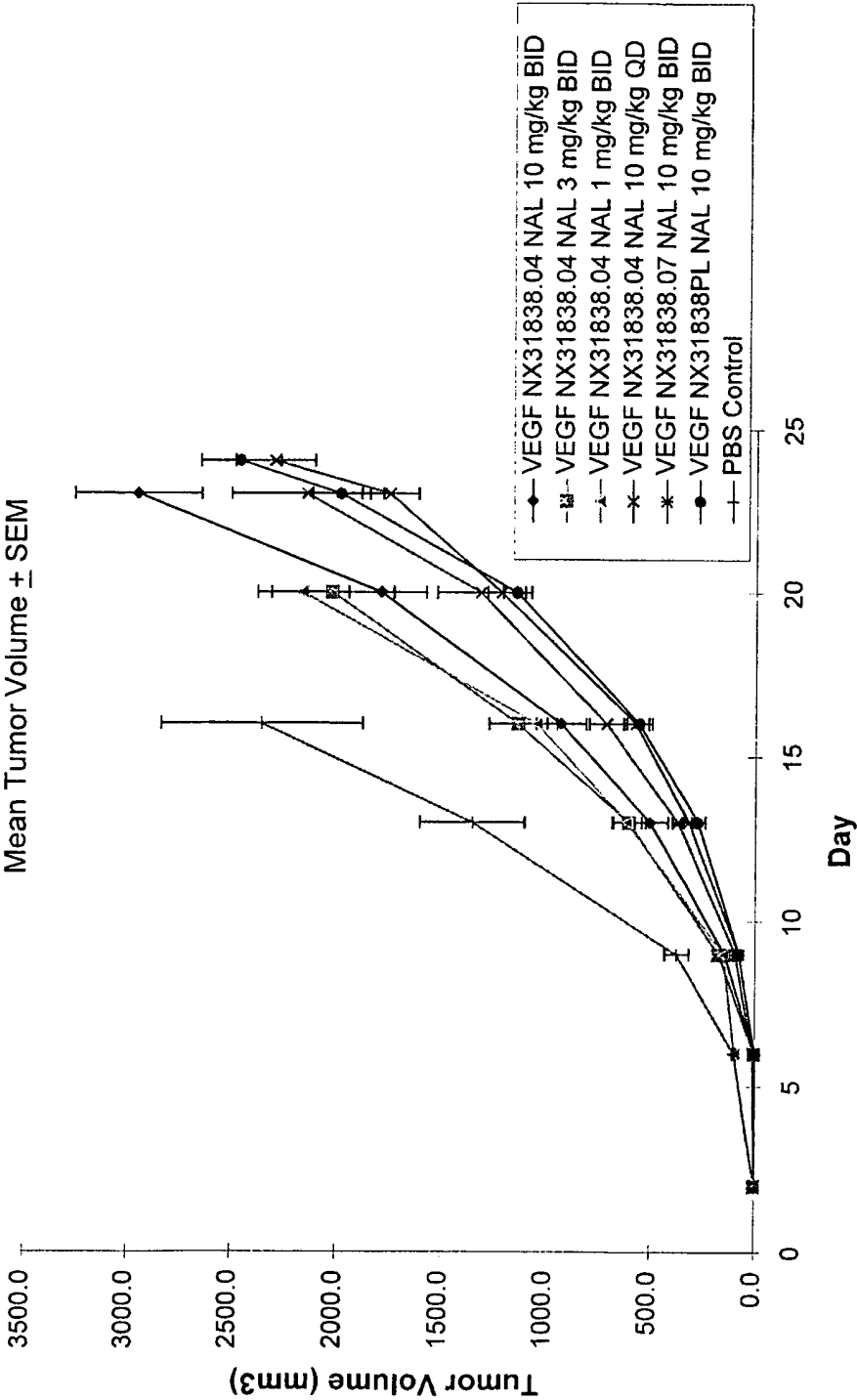
Figure 11

Mean Tumor Volume  $\pm$  SEM



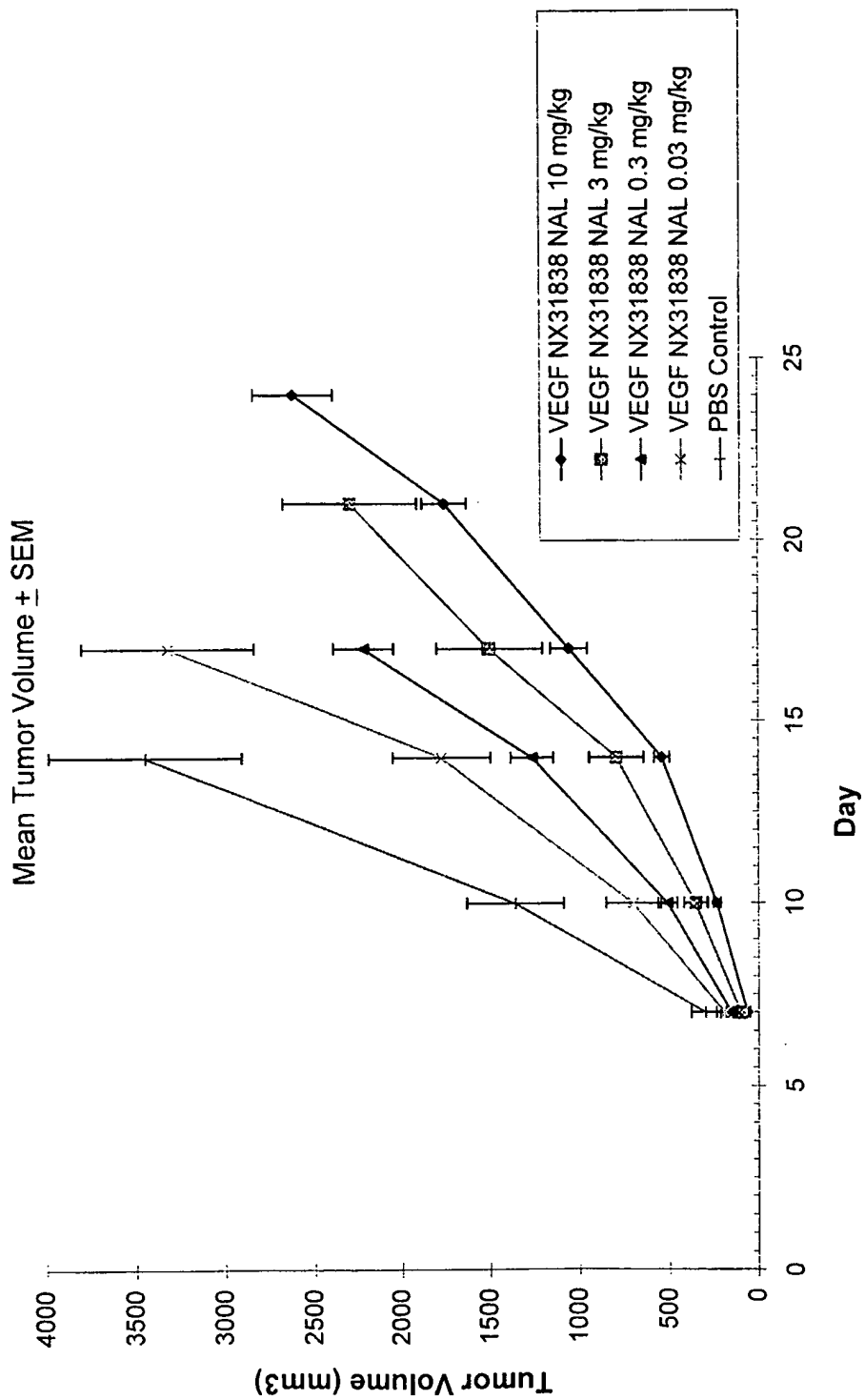
25/34

Figure 12



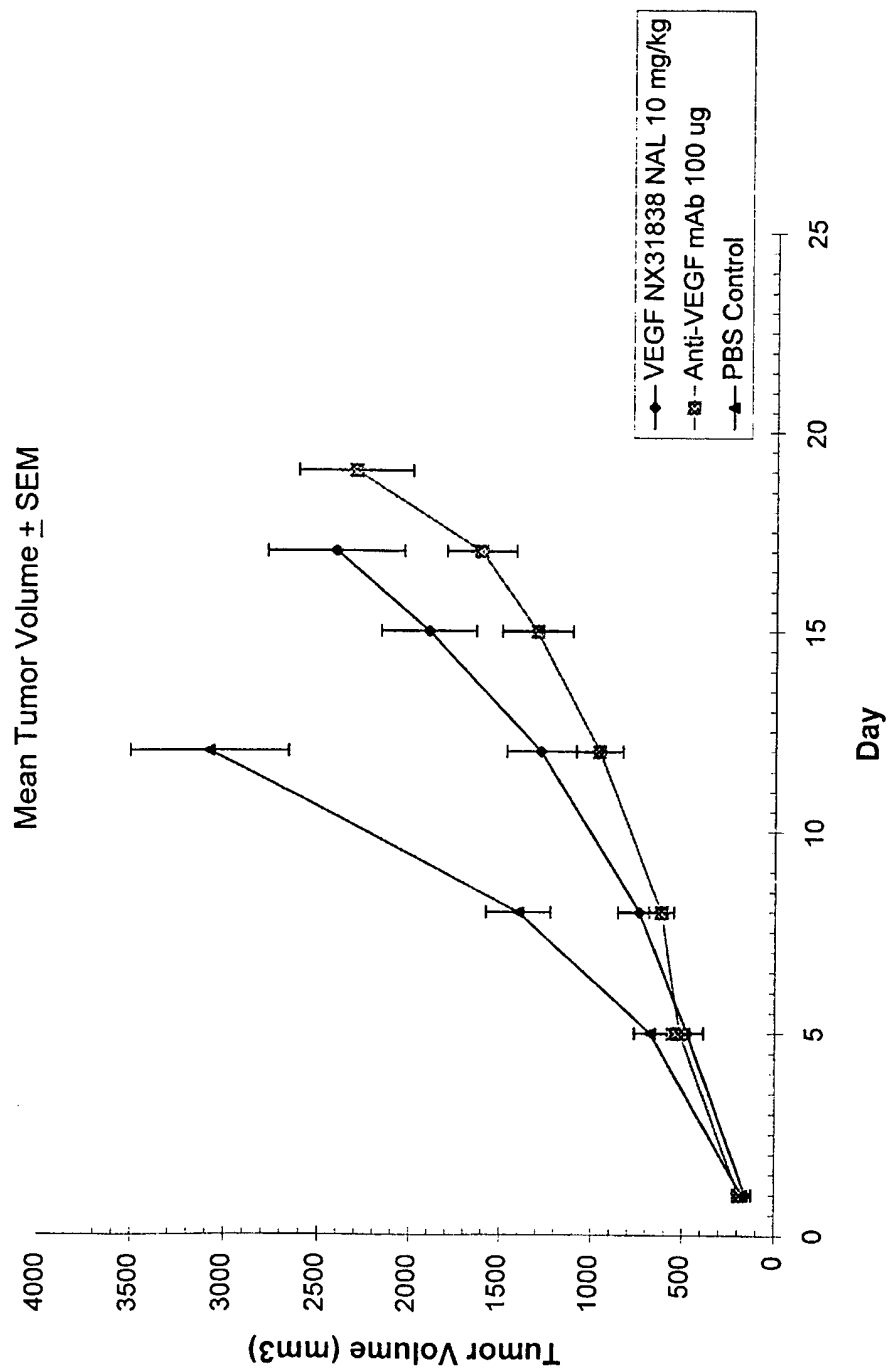
30/3A

Figure 13



3/1/3A

Figure 14



32/34

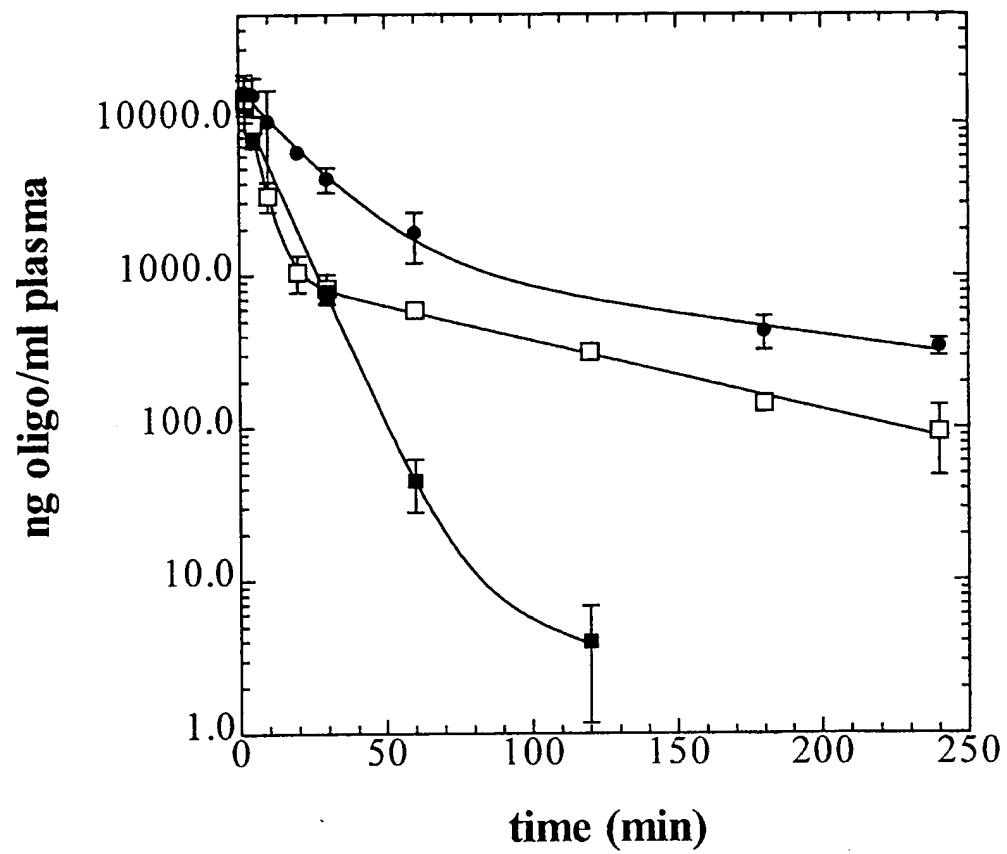


FIG. 15

33/3A

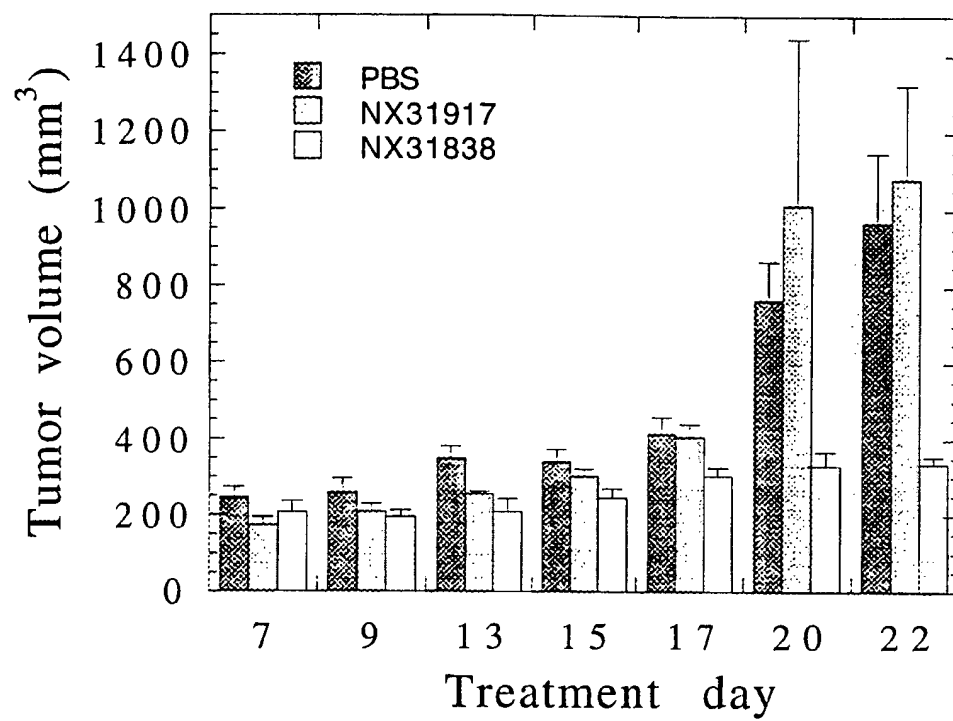


FIG. 16

3A/3A

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/18944

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : Please See Extra Sheet.

US CL : 424/450; 435/6, 91.2; 514/44; 536/22.1; 25.4

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/450; 435/6, 91.2; 514/44; 536/22.1; 25.4

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS; DIALOG ONESEARCH; NUCLEIC ACID LIGAND, APTAMER, LIPOSOME, LIPID CONJUGATE

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,459,015 A (JANJIC et al.) 17 October 1995, column 14, line 1 - column 16 line 16; column 16, Example 4; column 17, Example 5; column 6, line 34-column 8, line 13	1-84
Y	JAKEMAN, L.B. et al. Binding Sites for Vascular Endothelial Growth Factor Are Localized on Endothelial Cells in Adult Rat Tissues. J. Clin. Invest. January, 1992. Vol. 89, pages 244-252, especially page 244.	1-84
Y	WO 90/10448 A2 (GENENTECH, INC.) 20 September 1990, pages 4-6, 10-12, 14-16.	7-84



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

17 DECEMBER 1997

Date of mailing of the international search report

29 JAN 1998

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

STEPHANIE W. ZITOMER

Telephone No. (703) 308-0196

**INTERNATIONAL SEARCH REPORT**

International application No.  
PCT/US97/18944

**A. CLASSIFICATION OF SUBJECT MATTER:**

IPC (6):

A61K 37/22, 31/70, 47/48; C07H 21/02, 21/04; C12P 19/34; C12Q 1/68





## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>C07H 21/04, 21/02, C12Q 1/68, C12P 19/34</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 99/31119</b> <b>(43) International Publication Date:</b> 24 June 1999 (24.06.99)
<b>(21) International Application Number:</b> PCT/US98/09050 <b>(22) International Filing Date:</b> 29 April 1998 (29.04.98) <b>(30) Priority Data:</b> 08/991,743      16 December 1997 (16.12.97)      US <b>(71) Applicant:</b> NEXSTAR PHARMACEUTICALS, INC. [US/US]; Suite 200, 2860 Wilderness Place, Boulder, CO 80301 (US). <b>(72) Inventors:</b> JANJIC, Nebojsa; 6973 Carter Trail, Boulder, CO 80301 (US). GOLD, Larry; 1033 5th Street, Boulder, CO 80302 (US). <b>(74) Agents:</b> SWANSON, Barry, J. et al.; Swanson & Bratschun, L.L.C., Suite 200, 8400 E. Prentice Avenue, Englewood, CO 80111 (US).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> PLATELET DERIVED GROWTH FACTOR (PDGF) NUCLEIC ACID LIGAND COMPLEXES		
<b>(57) Abstract</b>  This invention discloses a method for preparing a complex comprised of a PDGF Nucleic Acid Ligand and a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound by identifying a PDGF Nucleic Acid Ligand by SELEX methodology and associating the PDGF Nucleic Acid Ligand with a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound. The invention further discloses Complexes comprising one or more PDGF Nucleic Acid Ligands in association with a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound. The invention further includes a Lipid construct comprising a PDGF Nucleic Acid Ligand or Complex and methods for making the same.		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav	TM	Turkmenistan
BF	Burkina Faso	GR	Greece		Republic of Macedonia	TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's	NZ	New Zealand		
CM	Cameroon		Republic of Korea	PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakhstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

**PLATELET DERIVED GROWTH FACTOR (PDGF)  
NUCLEIC ACID LIGAND COMPLEXES**

**5    FIELD OF THE INVENTION**

Described herein are high affinity ssDNA and RNA ligands to platelet derived growth factor (PDGF). The method utilized herein for identifying such Nucleic Acid Ligands is called SELEX, an acronym for Systematic Evolution of Ligands by Exponential enrichment. Further included in this invention is a method for preparing a therapeutic or diagnostic

10    Complex comprised of a PDGF Nucleic Acid Ligand and a Non-Immunogenic, High Molecular Weight Compound or a Lipophilic Compound by identifying a PDGF Nucleic Acid Ligand by SELEX methodology and covalently linking the PDGF Nucleic Acid Ligand with a Non-Immunogenic, High Molecular Weight Compound or a Lipophilic Compound. The invention further includes Complexes comprised of one or more PDGF Nucleic Acid Ligands

15    and a Non-Immunogenic, High Molecular Weight Compound or a Lipophilic Compound. The invention further relates to improving the Pharmacokinetic Properties of a PDGF Nucleic Acid Ligand by covalently linking the PDGF Nucleic Acid Ligand with a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound to form a Complex. The invention further relates to improving the Pharmacokinetic Properties of a PDGF Nucleic Acid

20    Ligand by using a Lipid Construct comprising a PDGF Nucleic Acid Ligand or a Complex comprising a PDGF Nucleic Acid Ligand and a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound. This invention further relates to a method for targeting a therapeutic or diagnostic agent to a biological target that is expressing PDGF by associating the agent with a Complex comprised of a PDGF Nucleic Acid Ligand and a Lipophilic

25    Compound or Non-Immunogenic, High Molecular Weight Compound, wherein the Complex is further associated with a Lipid Construct and the PDGF Nucleic Acid Ligand is further associated with the exterior of the Lipid Construct.

**BACKGROUND OF THE INVENTION**

30    **A. The SELEX Process**

The dogma for many years was that nucleic acids had primarily an informational role. Through a method known as Systematic Evolution of Ligands by Exponential enrichment, termed SELEX, it has become clear that nucleic acids have three dimensional structural diversity not unlike proteins. SELEX is a method for the *in vitro* evolution of nucleic acid

molecules with highly specific binding to target molecules and is described in United States Patent Application Serial No. 07/536,428, filed June 11, 1990, entitled "Systematic Evolution of Ligands by Exponential Enrichment," now abandoned, United States Patent Application Serial No. 07/714,131, filed June 10, 1991, entitled "Nucleic Acid Ligands," now United States Patent No. 5,475,096, United States Patent Application Serial No. 07/931,473, filed August 17, 1992, entitled "Methods for Identifying Nucleic Acid Ligands," now United States Patent No. 5,270,163 (see also WO 91/19813), each of which is specifically incorporated by reference herein. Each of these applications, collectively referred to herein as the SELEX Patent Applications, describes a fundamentally novel method for making a Nucleic Acid Ligand to any desired target molecule. The SELEX process provides a class of products which are referred to as Nucleic Acid Ligands, each ligand having a unique sequence, and which has the property of binding specifically to a desired target compound or molecule. Each SELEX-identified Nucleic Acid Ligand is a specific ligand of a given target compound or molecule. SELEX is based on the unique insight that Nucleic Acids have sufficient capacity for forming a variety of two- and three-dimensional structures and sufficient chemical versatility available within their monomers to act as ligands (form specific binding pairs) with virtually any chemical compound, whether monomeric or polymeric. Molecules of any size or composition can serve as targets.

The SELEX method involves selection from a mixture of candidate oligonucleotides and step-wise iterations of binding, partitioning and amplification, using the same general selection scheme, to achieve virtually any desired criterion of binding affinity and selectivity. Starting from a mixture of Nucleic Acids, preferably comprising a segment of randomized sequence, the SELEX method includes steps of contacting the mixture with the target under conditions favorable for binding, partitioning unbound Nucleic Acids from those Nucleic Acids which have bound specifically to target molecules, dissociating the Nucleic Acid-target complexes, amplifying the Nucleic Acids dissociated from the Nucleic Acid-target complexes to yield a ligand-enriched mixture of Nucleic Acids, then reiterating the steps of binding, partitioning, dissociating and amplifying through as many cycles as desired to yield highly specific high affinity Nucleic Acid Ligands to the target molecule.

It has been recognized by the present inventors that the SELEX method demonstrates that Nucleic Acids as chemical compounds can form a wide array of shapes, sizes and

configurations, and are capable of a far broader repertoire of binding and other functions than those displayed by Nucleic Acids in biological systems.

The present inventors have recognized that SELEX or SELEX-like processes could be used to identify Nucleic Acids which can facilitate any chosen reaction in a manner similar to that in which Nucleic Acid Ligands can be identified for any given target. In theory, within a Candidate Mixture of approximately  $10^{13}$  to  $10^{18}$  Nucleic Acids, the present inventors postulate that at least one Nucleic Acid exists with the appropriate shape to facilitate each of a broad variety of physical and chemical interactions.

The basic SELEX method has been modified to achieve a number of specific objectives. For example, United States Patent Application Serial No. 07/960,093, filed October 14, 1992, entitled "Method for Selecting Nucleic Acids on the Basis of Structure," describes the use of SELEX in conjunction with gel electrophoresis to select Nucleic Acid molecules with specific structural characteristics, such as bent DNA. United States Patent Application Serial No. 08/123,935, filed September 17, 1993, entitled "Photoselection of Nucleic Acid Ligands," describes a SELEX based method for selecting Nucleic Acid Ligands containing photoreactive groups capable of binding and/or photocrosslinking to and/or photoinactivating a target molecule. United States Patent Application Serial No. 08/134,028, filed October 7, 1993, entitled "High-Affinity Nucleic Acid Ligands That Discriminate Between Theophylline and Caffeine," abandoned in favor of United States Patent Application Serial No. 08/443,957, now United States Patent No. 5,580,737, describes a method for identifying highly specific Nucleic Acid Ligands able to discriminate between closely related molecules, which can be non-peptidic, termed Counter-SELEX. United States Patent Application Serial No. 08/143,564, filed October 25, 1993, entitled "Systematic Evolution of Ligands by Exponential Enrichment: Solution SELEX," abandoned in favor of United States Patent Application Serial No. 08/461,069, now United States Patent No. 5,567,588, describes a SELEX-based method which achieves highly efficient partitioning between oligonucleotides having high and low affinity for a target molecule.

The SELEX method encompasses the identification of high-affinity Nucleic Acid Ligands containing modified nucleotides conferring improved characteristics on the ligand, such as improved *in vivo* stability or improved delivery characteristics. Examples of such modifications include chemical substitutions at the ribose and/or phosphate and/or base positions. SELEX-identified Nucleic Acid Ligands containing modified nucleotides are

described in United States Patent Application Serial No. 08/117,991, filed September 8, 1993, entitled "High Affinity Nucleic Acid Ligands Containing Modified Nucleotides," abandoned in favor of United States Patent Application Serial No. 08/430,709, now United States Patent No. 5,660,985, that describes oligonucleotides containing nucleotide derivatives chemically modified at the 5- and 2'-positions of pyrimidines. United States Patent Application Serial No. 08/134,028, *supra*, describes highly specific Nucleic Acid Ligands containing one or more nucleotides modified with 2'-amino (2'-NH<sub>2</sub>), 2'-fluoro (2'-F), and/or 2'-O-methyl (2'-OMe). United States Patent Application Serial No. 08/264,029, filed June 22, 1994, entitled "Novel Method of Preparation of Known and Novel 2'-Modified Nucleosides by Intramolecular Nucleophilic Displacement," describes oligonucleotides containing various 2'-modified pyrimidines.

The SELEX method encompasses combining selected oligonucleotides with other selected oligonucleotides and non-oligonucleotide functional units as described in United States Patent Application Serial No. 08/284,063, filed August 2, 1994, entitled "Systematic Evolution of Ligands by Exponential Enrichment: Chimeric SELEX," now United States Patent No. 5,637,459, and United States Patent Application Serial No. 08/234,997, filed April 28, 1994, entitled "Systematic Evolution of Ligands by Exponential Enrichment: Blended SELEX," now United States Patent No. 5,683,867, respectively. These applications allow the combination of the broad array of shapes and other properties, and the efficient amplification and replication properties, of oligonucleotides with the desirable properties of other molecules.

The SELEX method further encompasses combining selected nucleic acid ligands with lipophilic compounds or non-immunogenic, high molecular weight compounds in a diagnostic or therapeutic complex as described in United States Patent Application Serial No. 08/434,465, filed May 4, 1995, entitled "Nucleic Acid Ligand Complexes." VEGF Nucleic Acid Ligands that are associated with a Lipophilic Compound, such as diacyl glycerol or dialkyl glycerol, in a diagnostic or therapeutic complex are described in United States Patent Application Serial No. 08/739,109, filed October 25, 1996, entitled "Vascular Endothelial Growth Factor (VEGF) Nucleic Acid Ligand Complexes." VEGF Nucleic Acid Ligands that are associated with a Lipophilic Compound, such as a glycerol lipid, or a Non-Immunogenic, High Molecular Weight Compound, such as polyethylene glycol, are further described in United States Patent Application Serial No. 08/897,351, filed July 21, 1997, entitled "Vascular Endothelial Growth Factor (VEGF) Nucleic Acid Ligand Complexes." VEGF Nucleic Acid

Ligands that are associated with a non-immunogenic, high molecular weight compound or lipophilic compound are also further described in PCT/US97/18944, filed October 17, 1997, entitled "Vascular Endothelial Growth Factor (VEGF) Nucleic Acid Ligand Complexes."

Each of the above described patent applications which describe modifications of the basic  
5 SELEX procedure are specifically incorporated by reference herein in their entirety.

## B. LIPID CONSTRUCTS

Lipid Bilayer Vesicles are closed, fluid-filled microscopic spheres which are formed principally from individual molecules having polar (hydrophilic) and non-polar (lipophilic)  
10 portions. The hydrophilic portions may comprise phosphato, glycerylphosphato, carboxy, sulfato, amino, hydroxy, choline or other polar groups. Examples of lipophilic groups are saturated or unsaturated hydrocarbons such as alkyl, alkenyl or other lipid groups. Sterols (e.g., cholesterol) and other pharmaceutically acceptable adjuvants (including anti-oxidants like alpha-tocopherol) may also be included to improve vesicle stability or confer other  
15 desirable characteristics.

Liposomes are a subset of these bilayer vesicles and are comprised principally of phospholipid molecules that contain two hydrophobic tails consisting of fatty acid chains. Upon exposure to water, these molecules spontaneously align to form spherical, bilayer  
20 membranes with the lipophilic ends of the molecules in each layer associated in the center of the membrane and the opposing polar ends forming the respective inner and outer surface of the bilayer membrane(s). Thus, each side of the membrane presents a hydrophilic surface while the interior of the membrane comprises a lipophilic medium. These membranes may be arranged in a series of concentric, spherical membranes separated by thin strata of water, in a manner not dissimilar to the layers of an onion, around an internal aqueous space.  
25 These multilamellar vesicles (MLV) can be converted into small or Unilamellar Vesicles (UV), with the application of a shearing force.

The therapeutic use of liposomes includes the delivery of drugs which are normally toxic in the free form. In the liposomal form, the toxic drug is occluded, and may be directed away from the tissues sensitive to the drug and targeted to selected areas.  
30 Liposomes can also be used therapeutically to release drugs over a prolonged period of time, reducing the frequency of administration. In addition, liposomes can provide a method for forming aqueous dispersions of hydrophobic or amphiphilic drugs, which are normally

unsuitable for intravenous delivery.

In order for many drugs and imaging agents to have therapeutic or diagnostic potential, it is necessary for them to be delivered to the proper location in the body, and the liposome can thus be readily injected and form the basis for sustained release and drug  
5 delivery to specific cell types, or parts of the body. Several techniques can be employed to use liposomes to target encapsulated drugs to selected host tissues, and away from sensitive tissues. These techniques include manipulating the size of the liposomes, their net surface charge, and their route of administration. MLVs, primarily because they are relatively large,  
10 are usually rapidly taken up by the reticuloendothelial system (principally the liver and spleen). UVs, on the other hand, have been found to exhibit increased circulation times, decreased clearance rates and greater biodistribution relative to MLVs.

Passive delivery of liposomes involves the use of various routes of administration, e.g., intravenous, subcutaneous, intramuscular and topical. Each route produces differences in localization of the liposomes. Two common methods used to direct liposomes actively to  
15 selected target areas involve attachment of either antibodies or specific receptor ligands to the surface of the liposomes. Antibodies are known to have a high specificity for their corresponding antigen and have been attached to the surface of liposomes, but the results have been less than successful in many instances. Some efforts, however, have been successful in targeting liposomes to tumors without the use of antibodies, see, for example,  
20 U.S. Patent No. 5,019,369, U.S. Patent No. 5,441,745, or U.S. Patent No. 5,435,989.

An area of development aggressively pursued by researchers is the delivery of agents not only to a specific cell type but into the cell's cytoplasm and, further yet, into the nucleus. This is particularly important for the delivery of biological agents such as DNA, RNA, ribozymes and proteins. A promising therapeutic pursuit in this area involves the use of  
25 antisense DNA and RNA oligonucleotides for the treatment of disease. However, one major problem encountered in the effective application of antisense technology is that oligonucleotides in their phosphodiester form are quickly degraded in body fluids and by intracellular and extracellular enzymes, such as endonucleases and exonucleases, before the target cell is reached. Intravenous administration also results in rapid clearance from the  
30 bloodstream by the kidney, and uptake is insufficient to produce an effective intracellular drug concentration. Liposome encapsulation protects the oligonucleotides from the degradative enzymes, increases the circulation half-life and increases uptake efficiency as a



result of phagocytosis of the Liposomes. In this way, oligonucleotides are able to reach their desired target and to be delivered to cells *in vivo*.

A few instances have been reported where researchers have attached antisense oligonucleotides to Lipophilic Compounds or Non-Immunogenic, High Molecular Weight Compounds. Antisense oligonucleotides, however, are only effective as intracellular agents. Antisense oligodeoxyribonucleotides targeted to the epidermal growth factor (EGF) receptor have been encapsulated into Liposomes linked to folate via a polyethylene glycol spacer (folate-PEG-Liposomes) and delivered into cultured KB cells via folate receptor-mediated endocytosis (Wang *et al.* Proc. Natl. Acad. Sci. USA (1995) 92:3318-3322). In addition, alkylene diols have been attached to oligonucleotides (Weiss *et al.*, U.S. Patent No. 5,245,022). Furthermore, a Lipophilic Compound covalently attached to an antisense oligonucleotide has been demonstrated in the literature (EP 462 145 B1).

Loading of biological agents into liposomes can be accomplished by inclusion in the lipid formulation or loading into preformed liposomes. Passive anchoring of oligopeptide and oligosaccharide ligands to the external surface of liposomes has been described (Zalipsky *et al.* (1997) Bioconjug. Chem. 8:111:118).

### C. PDGF

Platelet-derived growth factor (PDGF) was originally isolated from platelet lysates and identified as the major growth-promoting activity present in serum but not in plasma. Two homologous PDGF isoforms have been identified, PDGF A and B, which are encoded by separate genes (on chromosomes 7 and 22). The most abundant species from platelets is the AB heterodimer, although all three possible dimers (AA, AB and BB) occur naturally. Following translation, PDGF dimers are processed into  $\approx 30$  kDa secreted proteins. Two cell surface proteins that bind PDGF with high affinity have been identified,  $\alpha$  and  $\beta$  (Heldin *et al.* (1981) Proc. Natl. Acad. Sci. 78:3664; Williams *et al.* (1981) Proc. Natl. Acad. Sci. 79:5867). Both species contain five immunoglobulin-like extracellular domains, a single transmembrane domain and an intracellular tyrosine kinase domain separated by a kinase insert domain. The functional high affinity receptor is a dimer and engagement of the extracellular domain of the receptor by PDGF results in cross-phosphorylation (one receptor tyrosine kinase phosphorylates the other in the dimer) of several tyrosine residues. Receptor phosphorylation leads to a cascade of events that results in the transduction of the

mitogenic or chemotactic signal to the nucleus. For example, in the intracellular domain of the PDGF  $\beta$  receptor, nine tyrosine residues have been identified that when phosphorylated interact with different src-homology 2 (SH2) domain-containing proteins including phospholipase C-g, phosphatidylinositol 3'-kinase, GTPase-activating protein and several adapter molecules like Shc, Grb2 and Nck (Heldin (1995) Cell 80:213). In the last several years, the specificities of the three PDGF isoforms for the three receptor dimers ( $\alpha\alpha$ ,  $\alpha\beta$ , and  $\beta\beta$ ) has been elucidated. The  $\alpha$ -receptor homodimer binds all three PDGF isoforms with high affinity, the  $\beta$ -receptor homodimer binds only PDGF BB with high affinity and PDGF-AB with approximately 10-fold lower affinity, and the  $\alpha\beta$ -receptor heterodimer binds PDGF-BB and PDGF-AB with high affinity (Westermarck & Heldin (1993) Acta Oncologica 32:101). The specificity pattern results from the ability of the A-chain to bind only to the  $\alpha$ -receptor and of the B-chain to bind to both  $\alpha$  and  $\beta$ -receptor subunits with high affinity.

The role of PDGF in proliferative diseases, such as cancer, restenosis, fibrosis, angiogenesis, and wound healing has been established.

#### PDGF in Cancer

The earliest indication that PDGF expression is linked to malignant transformation came with the finding that the amino acid sequence of PDGF-B chain is virtually identical to that of p28<sup>sis</sup>, the transforming protein of the simian sarcoma virus (SSV) (Waterfield *et al.* (1983) Nature 304:35; Johnsson *et al.* (1984) EMBO J. 3:921). The transforming potential of the PDGF-B chain gene and, to a lesser extent, the PDGF-A gene was demonstrated soon thereafter (Clarke *et al.* (1984) Nature 308:464; Gazit *et al.* (1984) Cell 39:89; Beckmann *et al.* Science 241:1346; Bywater *et al.* (1988) Mol. Cell. Biol. 8:2753). Many tumor cell lines have since been shown to produce and secrete PDGF, some of which also express PDGF receptors (Raines *et al.* (1990) in Peptide Growth Factors and Their Receptors, Springer-Verlag, Part I, p 173). Paracrine and, in some cell lines, autocrine growth stimulation by PDGF is therefore possible. For example, analysis of biopsies from human gliomas has revealed the existence of two autocrine loops: PDGF-B/ $\beta$ -receptor in tumor-associated endothelial cells and PDGF-A/ $\alpha$ -receptor in tumor cells (Hermansson *et al.* (1988) Proc. Natl. Acad. Sci. USA 85:7748; Hermansson *et al.* (1992) Cancer Res. 52:3213). The progression to high grade glioma was accompanied by the increase in expression of PDGF-B and the  $\beta$ -receptor in tumor-associated endothelial cells and

PDGF-A in glioma cells. PDGF overexpression may thus promote tumor growth either by directly stimulating tumor cells or by stimulating tumor-associated stromal cells (e.g., endothelial cells). The proliferation of endothelial cells is a hallmark of angiogenesis. Increased expression of PDGF and/or PDGF receptors has also been observed in other malignancies including fibrosarcoma (Smits *et al.* (1992) *Am. J. Pathol.* 140:639) and thyroid carcinoma (Heldin *et al.* (1991) *Endocrinology* 129:2187).

#### PDGF in Cardiovascular Disease

Percutaneous transluminal coronary angioplasty (PTCA) has become the most common treatment for occlusive coronary artery disease (CAD) involving one or two coronary arteries. In the United States alone about 500,000 procedures are being done annually, with projections of over 700,000 procedures by the year 2000 and about double those amounts worldwide. PTCA, while it involves manipulations inside of coronary arteries, is not considered to be a cardiac surgical intervention. During the most common PTCA procedure, a balloon catheter is threaded through a femoral artery and is positioned within the plaque-laden segment of an occluded coronary vessel; once in place, the balloon is expanded at high pressure, compressing the plaque and increasing the vessel lumen. Unfortunately, in 30-50% of PTCA procedures, reocclusion gradually develops over a period of several weeks or months due to cellular events in the affected vessel wall. Once reocclusion achieves 50% or greater reduction of the original vessel lumen, clinical restenosis is established in the vessel.

In view of the increasing popularity of coronary angioplasty as a less invasive alternative to bypass surgery, restenosis is a serious medical problem. Smooth muscle cells (SMCs) represent a major component of the restenosis lesions. In uninjured arteries, SMCs reside primarily in the medial vessel layer (tunica media). Upon balloon injury that removes the endothelial cells from the intimal layer (tunica intima), SMCs proliferate and migrate into the intima, forming neointimal thickening characteristic of restenosis lesions. When restenosis occurs subsequent to angioplasty, it is usually treated by repeat angioplasty, with or without placement of a stent, or by vascular graft surgery (bypass).

A stent is a rigid cylindrical mesh that, once placed and expanded within a diseased vessel segment, mechanically retains the expanded vessel wall. The stent is deployed by catheter and, having been positioned at the desired site, is expanded in situ by inflation of a high pressure balloon. Being rigid and non-compressible, the expanded stent achieves and

maintains a vessel lumen diameter comparable to that of adjacent non-diseased vessel; being pressed tightly into the overlying intima/media, it is resistant to migration within the vessel in response to blood flow. PTCA with stent placement has been compared with PTCA alone and shown to reduce restenosis to about half and to significantly improve other clinical outcomes such as myocardial infarction (MI) and need for bypass surgery.

There is now considerable evidence that PDGF-B-chain is a major contributor to the formation of neointimal lesions. In a rat model of restenosis, the neointimal thickening was inhibited with anti-PDGF-B antibodies (Ferns (1991) *Science* 253:1129-1132; Rutherford *et al.* (1997) *Atherosclerosis* 130:45-51). Conversely, the exogenous administration of PDGF-BB promotes SMC migration and causes an increase in neointimal thickening (Jawien *et al.* (1992) *J. Clin. Invest.* 89:507-511). The effect of PDGF-B on SMCs is mediated through PDGF  $\beta$ -receptor which is expressed at high levels in these cells after balloon injury (Lindner and Reidy (1995) *Circulation Res.* 76:951-957). Furthermore, the degree of neointimal thickening following balloon injury was found to be inversely related to the level of expression of PDGF  $\beta$ -receptor at the site of injury (Sirois *et al.* (1997) *Circulation* 95:669-676).

United States Patent No. 5,171,217 discloses a method and composition for delivery of a drug to an affected intramural site for sustained release in conjunction with or following balloon catheter procedures, such as angioplasty. The drug may be selected from a variety of drugs known to inhibit smooth muscle cell proliferation, including growth factor receptor antagonists for PDGF.

United States Patent No. 5,593,974 discloses methods for treating vascular disorders, such as vascular restenosis, with antisense oligonucleotides. The method is based on localized application of the antisense oligonucleotides to a specific site in vivo. The oligonucleotides can be applied directly to the target tissue in a mixture with an implant or gel, or by direct injection or infusion.

United States Patent No. 5,562,922 discloses a method for preparing a system suitable for localized delivery of biologically active compounds to a subject. The method relates to treating polyurethane coated substrate with a coating expansion solution under conditions that will allow penetration of the biologically active compound throughout the polyurethane coating. Substrates suitable for this invention include, inter alia, metallic

stents. Biologically active compounds suitable for use in this invention include, inter alia, lipid-modified oligonucleotides.

Rutherford *et al.* (1997, *Atherosclerosis* 130:45-51) report substantial inhibition of neointimal response to balloon injury in rat carotid artery using a combination of  
5 antibodies to PDGF-BB and basic fibroblast growth factor (bFGF).

#### PDGF in Renal Disease

A large variety of progressive renal diseases are characterized by glomerular mesangial cell proliferation and matrix accumulation (Slomowitz *et al.* (1988) *New Eng. J. Med.* 319:1547-1548) which leads to fibrosis. PDGF-B-chain appears to have a central role  
10 in driving both of these processes given that 1) mesangial cells produce PDGF *in vitro* and various growth factors induce mesangial proliferation via induction of auto- or paracrine PDGF-B-chain synthesis; 2) PDGF B-chain and its receptor are overexpressed in many glomerular diseases; 3) infusion of PDGF-BB or glomerular transfection with a PDGF-B-chain cDNA can induce selective mesangial cell proliferation and matrix accumulation *in*  
15 *vivo*; and 4) PDGF-B-chain or  $\beta$ -receptor knock-out mice fail to develop a mesangium (reviewed in Floege and Johnson (1995) *Miner. Electrolyte Metab.* 21:271-282). In addition to contributing to kidney fibrosis, PDGF is also believed to play a role in fibrosis development in other organs such as lungs and bone marrow and may have other possible disease associations (Raines *et al.* (1990) in Experimental Pharmacology, Peptide Growth  
20 Factors and Their Receptors, Sporn & Roberts, eds., pp. 173-262, Springer, Heidelberg).

One study has examined the effect of inhibition of PDGF-B-chain in renal disease: Johnson *et al.*, using a neutralizing polyclonal antibody to PDGF, were able to reduce mesangial cell proliferation and matrix accumulation in experimental mesangioproliferative glomerulonephritis (Johnson *et al.* (1992) *J. Exp. Med.* 175:1413-1416). In this model,  
25 injection of an anti-mesangial cell antibody (anti-Thy 1.1) into rats resulted in complement-dependent lysis of the mesangial cells, followed by an overshooting reparative phase that resembled human mesangioproliferative nephritis (Floege *et al.* (1993) *Kidney Int. Suppl.* 39:S47-54). Limitations of the study of Johnson *et al.* (Johnson *et al.* (1992) *J. Exp. Med.* 175:1413-1416) included the necessity to administer large amounts of heterologous IgG and  
30 a limitation of the study duration to 4 days due to concerns that the heterologous IgG might elicit an immune reaction.

### Inhibition of PDGF

Specific inhibition of growth factors, such as PDGF, has become a major goal in experimental and clinical medicine. However, this approach is usually hampered by the lack of specific pharmacological antagonists. Available alternative approaches are also limited, since neutralizing antibodies often show a low efficacy *in vivo* and are usually immunogenic, and given that *in vivo* gene therapy for these purposes is still in its infancy. Currently, antibodies to PDGF (Johnsson *et al.* (1985) Proc. Natl. Acad. Sci. USA 82:1721-1725; Ferns *et al.* (1991) Science 253:1129-1132; Herren *et al.* (1993) Biochimica et Biophysica Acta 1173:294-302; Rutherford *et al.* (1997) Atherosclerosis 130:45-51) and the soluble PDGF receptors (Herren *et al.* (1993) Biochimica et Biophysica Acta 1173:294-302; Duan *et al.* (1991) J. Biol. Chem. 266:413-418; Tiesman *et al.* (1993) J. Biol. Chem. 268:9621-9628) are the most potent and specific antagonists of PDGF. Neutralizing antibodies to PDGF have been shown to revert the SSV-transformed phenotype (Johnsson *et al.* (1985) Proc. Natl. Acad. Sci. USA 82:1721-1725) and to inhibit the development of neointimal lesions following arterial injury (Ferns *et al.* (1991) Science 253:1129-1132). Other inhibitors of PDGF such as suramin (Williams *et al.* (1984) J. Biol. Chem. 259:287-5294; Betsholtz *et al.* (1984) Cell 39:447-457), neomycin (Vassbotn *et al.* (1992) J. Biol. Chem. 267:15635-15641) and peptides derived from the PDGF amino acid sequence (Engström *et al.* (1992) J. Biol. Chem. 267:16581-16587) have been reported, however, they are either too toxic or lack sufficient specificity or potency to be good drug candidates. Other types of antagonists of possible clinical utility are molecules that selectively inhibit the PDGF receptor tyrosine kinase (Buchdunger *et al.* (1995) Proc. Natl. Acad. Sci. USA 92:2558-2562; Kovalenko *et al.* (1994) Cancer Res. 54:6106-6114).

### 25 SUMMARY OF THE INVENTION

The present invention includes methods of identifying and producing nucleic acid ligands to platelet-derived growth factor (PDGF) and homologous proteins and the nucleic acid ligands so identified and produced. For the purposes of this application, PDGF refers to PDGF-AA, -AB, and -BB isoforms and homologous proteins. Specifically included in the definition are human PDGF-AA, -AB, and -BB isoforms.

Described herein are high affinity ssDNA and RNA ligands to platelet derived growth factor (PDGF). The method utilized herein for identifying such nucleic acid ligands is called

SELEX, an acronym for Systematic Evolution of Ligands by Exponential enrichment.

Included herein are the evolved ligands that are shown in Tables 2-3, 6-7, and 9 and Figures 1-2A, 2B, 2C, 8A, 8B and 9A. Further included in this invention is a method for preparing a Complex comprised of a PDGF Nucleic Acid Ligand and a Non-Immunogenic, High

5 Molecular Weight Compound or Lipophilic Compound by the method comprising identifying a Nucleic Acid Ligand from a Candidate Mixture of Nucleic Acids where the Nucleic Acid is a ligand of PDGF by the method of (a) contacting the Candidate Mixture of Nucleic Acids with PDGF, (b) partitioning between members of said Candidate Mixture on the basis of affinity to PDGF, and c) amplifying the selected molecules to yield a mixture of Nucleic Acids  
10 enriched for Nucleic Acid sequences with a relatively higher affinity for binding to PDGF, and covalently linking said identified PDGF Nucleic Acid Ligand with a Non-Immunogenic, High Molecular Weight Compound or a Lipophilic Compound. The invention further comprises a Complex comprised of a PDGF Nucleic Acid Ligand and a Non-Immunogenic, High Molecular Weight Compound or a Lipophilic Compound.

15 The invention further includes a Lipid Construct comprising a PDGF Nucleic Acid Ligand or a Complex. The present invention further relates to a method for preparing a Lipid Construct comprising a Complex wherein the Complex is comprised of a PDGF Nucleic Acid Ligand and a Lipophilic Compound.

In another embodiment, this invention provides a method for improving the  
20 pharmacokinetic properties of a PDGF Nucleic Acid Ligand by covalently linking the PDGF Nucleic Acid Ligand with a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound to form a Complex and administering the Complex to a patient. The invention further relates to a method for improving the pharmacokinetic properties of a PDGF Nucleic Acid Ligand by further associating the Complex with a Lipid Construct.

25 It is an object of the present invention to provide Complexes comprising one or more PDGF Nucleic Acid Ligands in association with one or more Non-Immunogenic, High Molecular Weight Compounds or Lipophilic Compounds and methods for producing the same. It is a further object of the present invention to provide Lipid Constructs comprising a Complex. It is a further object of the invention to provide one or more PDGF Nucleic Acid  
30 Ligands in association with one or more Non-Immunogenic, High Molecular Weight Compounds or Lipophilic Compounds with improved Pharmacokinetic Properties.

In embodiments of the invention directed to Complexes comprised of a PDGF Nucleic Acid Ligand and a Non-Immunogenic, High Molecular Weight Compound, it is preferred that the Non-Immunogenic, High Molecular Weight Compound is Polyalkylene Glycol, more preferably, polyethylene glycol (PEG). More preferably, the PEG has a molecular weight of about 10-80K. Most preferably, the PEG has a molecular weight of about 20-45K. In  
5       embodiments of the invention directed to Complexes comprised of a PDGF Nucleic Acid Ligand and a Lipophilic Compound, it is preferred that the Lipophilic Compound is a glycerolipid. In the preferred embodiments of the invention, the Lipid Construct is preferably a Lipid Bilayer Vesicle and most preferably a Liposome. In the preferred embodiment, the  
10       PDGF Nucleic Acid Ligand is identified according to the SELEX method.

In embodiments of the invention directed to Complexes comprising a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound covalently linked to a PDGF Nucleic Acid Ligand or Ligands, the PDGF Nucleic Acid Ligand or Ligands can serve in a targeting capacity.

15       Additionally, the PDGF Nucleic Acid Ligand can be associated through Covalent or Non-Covalent Interactions with a Lipid Construct without being part of a Complex.

Furthermore, in embodiments of the invention directed to Lipid Constructs comprising a PDGF Nucleic Acid Ligand or a Non-Immunogenic, High Molecular Weight or Lipophilic Compound/PDGF Nucleic Acid Ligand Complex where the Lipid Construct is of a type that  
20       has a membrane defining an interior compartment such as a Lipid Bilayer Vesicle, the PDGF Nucleic Acid Ligand or Complex in association with the Lipid Construct may be associated with the membrane of the Lipid Construct or encapsulated within the compartment. In embodiments where the PDGF Nucleic Acid Ligand is in association with the membrane, the PDGF Nucleic Acid Ligand can associate with the interior-facing or exterior-facing part of  
25       the membrane, such that the PDGF Nucleic Acid Ligand is projecting into or out of the vesicle. In certain embodiments, a PDGF Nucleic Acid Ligand Complex can be passively loaded onto the outside of a preformed Lipid Construct. In embodiments where the Nucleic Acid Ligand is projecting out of the Lipid Construct, the PDGF Nucleic Acid Ligand can serve in a targeting capacity.

30       In embodiments where the PDGF Nucleic Acid Ligand of the Lipid Construct serves in a targeting capacity, the Lipid Construct can have associated with it additional therapeutic or diagnostic agents. In one embodiment, the therapeutic or diagnostic agent is associated



with the exterior of the Lipid Construct. In other embodiments, the therapeutic or diagnostic agent is encapsulated in the Lipid Construct or associated with the interior of the Lipid Construct. In yet a further embodiment, the therapeutic or diagnostic agent is associated with the Complex. In one embodiment, the therapeutic agent is a drug. In an alternative  
5 embodiment, the therapeutic or diagnostic agent is one or more additional Nucleic Acid Ligands.

It is a further object of the present invention to provide a method for inhibiting PDGF-mediated diseases. PDGF-mediated diseases include, but are not limited to, cancer, angiogenesis, restenosis, and fibrosis. Thus, it is a further object of the present invention to  
10 provide a method for inhibiting angiogenesis by the administration of a PDGF Nucleic Acid Ligand or a Complex comprising a PDGF Nucleic Acid Ligand and Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound or a Lipid Construct comprising the Complex of the present invention. It is yet a further object of the present invention to provide a method for inhibiting the growth of tumors by the administration of a PDGF Nucleic Acid  
15 Ligand or Complex comprising a PDGF Nucleic Acid Ligand and Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound or a Lipid Construct comprising a Complex of the present invention. It is yet a further object of the invention to provide a method for inhibiting fibrosis by the administration of a PDGF Nucleic Acid Ligand or Complex comprising a PDGF Nucleic Acid Ligand and Non-Immunogenic, High Molecular  
20 Weight Compound or Lipophilic Compound or a Lipid Construct comprising a Complex of the present invention. It is yet a further object of the invention to provide a method for inhibiting restenosis by the administration of a PDGF Nucleic Acid Ligand or Complex comprising a PDGF Nucleic Acid Ligand and Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound or a Lipid Construct comprising a Complex of the  
25 present invention

It is a further object of the invention to provide a method for targeting a therapeutic or diagnostic agent to a biological target that is expressing PDGF by associating the agent with a Complex comprised of a PDGF Nucleic Acid Ligand and a Lipophilic Compound or Non-Immunogenic, High Molecular Weight Compound, wherein the Complex is further associated  
30 with a Lipid Construct and the PDGF Nucleic Acid Ligand is further associated with the exterior of the Lipid Construct.

These and other objects, as well as the nature, scope and utilization of this invention, will become readily apparent to those skilled in the art from the following description and the appended claims.

## 5 **BRIEF DESCRIPTION OF THE FIGURES**

Figure 1 shows the consensus secondary structure for the sequence set shown in Table 3. R = A or G, Y = C or T, K = G or T, N and N' indicate any base pair.

Figures 2A-2C show the minimal ligands 20t (SEQ ID NO:83), 36t (SEQ ID NO:84) and 41t (SEQ ID NO:85) folded according to the consensus secondary structure motif. [3'T] represents a 3'-3' linked thymidine nucleotide added to reduce 3'-exonuclease degradation.

Figures 3A-3C show the binding of minimal high affinity DNA ligands to PDGF-AA, PDGF-AB, and PDGF-BB, respectively. The fraction of  $^{32}\text{P}$  5' end-labeled DNA ligands bound to varying concentrations of PDGF was determined by the nitrocellulose filter binding method. Minimal ligands tested were 20t (o), 36t ( $\Delta$ ), and 41t ( $\square$ ).  
Oligonucleotide concentrations in these experiments were  $\approx 10$  pM (PDGF-AB and PDGF-BB) and  $\approx 50$  pM (PDGF-AA). Data points were fitted to equation 1 (for binding of the DNA ligands to PDGF-AA) or to equation 2 (for binding to PDGF-AB and -BB) using the non-linear least squares method. Binding reactions were done at  $37^\circ\text{C}$  in binding buffer (PBSM with 0.01% HSA).

Figure 4 shows the dissociation rate determination for the high affinity interaction between the minimal DNA ligands and PDGF-AB. The fraction of 5'  $^{32}\text{P}$  end-labeled ligands 20t (o), 36t ( $\Delta$ ), and 41t ( $\square$ ), all at 0.17 nM, bound to PDGF-AB (1 nM) was measured by nitrocellulose filter binding at the indicated time points following the addition of a 500-fold excess of the unlabeled competitor. The dissociation rate constant ( $k_{\text{off}}$ ) values were determined by fitting the data points to eq 3 in Example 1. The experiments were performed at  $37^\circ\text{C}$  in binding buffer.

Figure 5 shows the thermal denaturation profiles for the minimal high affinity DNA ligands to PDGF-AB. The change in absorbance at 260 nm was measured in PBS containing 1 mM  $\text{MgCl}_2$  as a function of temperature for ligands 20t (o), 36t ( $\Delta$ ), and 41t ( $\square$ ).

Figure 6 shows the effect of DNA ligands on the binding of  $^{125}\text{I}$ -PDGF-BB to PDGF  $\alpha$ -receptors expressed in PAE cells.

Figure 7 shows the effect of DNA ligands on the mitogenic effect of PDGF-BB on PAE cells expressing the PDGF  $\beta$ -receptors.

Figures 8A-8B show the substitution pattern compatible with high affinity binding to PDGF-AB. In Figures 8A-8C, the underlined symbols indicate

5 2'-O-methyl-2'-deoxynucleotides; italicized symbols indicate 2'-fluoro-2'-deoxynucleotides; normal font indicates 2'-deoxyribonucleotides; [3'T] indicates inverted orientation (3'3') thymidine nucleotide (Glen Research, Sterling, VA); PEG in the loops of helices II and III of Figure 8B indicates pentaethylene glycol spacer phosphoramidite (Glen Research, Sterling, VA) (See Figure 9 for molecular description). Figure 8C shows the predicted  
10 secondary structure of a scrambled Nucleic Acid Ligand sequence that was used as a control in Examples 8 and 9. The scrambled region is boxed to accent the overall similarity of the scrambled Nucleic Acid Ligand to the Nucleic Acid Ligand shown in Figure 8B.

Figures 9A-9E show the molecular descriptions NX31975-40K PEG (SEQ ID NO:146) (Figure 9A), NX31976-40K (SEQ ID NO:147) (Figure 9B), hexaethylene glycol  
15 phosphoramidite (Figure 9C), pentyl amino linker (Figure 9D), and 40K PEG NHS ester (Figure 9E). The 5' phosphate group shown in the PEG Spacer of Figures 9A and 9B are from the hexaethylene glycol phosphoramidite.

Figure 10 shows the stabilities of DNA (36ta) and modified DNA (NX21568) Nucleic Acid Ligands in rat serum over time at 37°C. 36ta is shown by the symbol ●; and  
20 NX21568 is shown by the symbol ▲.

Figure 11 shows that NX31975-40K PEG significantly inhibited ( $p < 0.05$ ) about 50% of the neointima formation in rats based on the intima/media ratio for the control (PBS) and NX31975-40K PEG groups.

Figure 12 shows the effects of NX31975-40K PEG on mitogen-stimulated  
25 proliferation of mesangial cells in culture (all mitogens were added at 100 ng/ml final concentration). Scrambled Nucleic Acid Ligand NX31976 and 40K PEG were also tested. Data are optical densities measured in the XTT assay and are expressed as percentages of baseline, i.e., cells stimulated with medium plus 200  $\mu$ g/ml 40K PEG (i.e., the amount equivalent to the PEG attached to 50  $\mu$ g/ml Nucleic Acid Ligand). Results are means  $\pm$  SD  
30 of 5 separate experiments ( $n = 3$  in the case of medium plus 40K PEG; statistical evaluation was therefore confined to NX31975 and scrambled Nucleic Acid Ligand groups).

Figures 13A-13E show effects of NX31975-40K PEG on glomerular cell

proliferation (Figure 13A), expression of glomerular PDGF B-chain (Figure 13B), proteinuria in rats with anti-Thy 1.1 nephritis (Figure 13C), mesangial cell activation (as assessed by glomerular *de novo* expression of  $\alpha$ -smooth muscle actin) (Figure 13D), and monocyte/macrophage influx (Figure 13E). NX31975-40K PEG is shown as black, NX31976-40K PEG is shown as cross-hatched, 40K PEG is shown as white, PBS is shown as hatched, and the normal range is shown as stippled.

Figures 14A-C show the effects of NX31975-40K PEG on glomerular matrix accumulation. Glomerular immunostaining scores for fibronectin and type IV collagen as well as glomerular scores for type IV collagen mRNA expression (*in situ* hybridization) are shown. NX31975-40K PEG is shown as black, NX31976-40K PEG is shown as cross-hatched, 40K PEG is shown as white, PBS is shown as hatched, and the normal range is shown as stippled.

## **DETAILED DESCRIPTION OF THE INVENTION**

### **DEFINITIONS:**

"**Covalent Bond**" is the chemical bond formed by the sharing of electrons.

"**Non-Covalent Interactions**" are means by which molecular entities are held together by interactions other than Covalent Bonds including ionic interactions and hydrogen bonds.

"**Lipophilic Compounds**" are compounds which have the propensity to associate with or partition into lipid and/or other materials or phases with low dielectric constants, including structures that are comprised substantially of lipophilic components. Lipophilic Compounds include lipids as well as non-lipid containing compounds that have the propensity to associate with lipid (and/or other materials or phases with low dielectric constants).

Cholesterol, phospholipids, and glycerolipids, such as dialkylglycerol, and diacylglycerol, and glycerol amide lipids are further examples of Lipophilic Compounds.

"**Complex**" as used herein describes the molecular entity formed by the covalent linking of a PDGF Nucleic Acid Ligand to a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound. In certain embodiments of the present invention, the Complex is depicted as A-B-Y, wherein A is a Lipophilic Compound or Non-Immunogenic, High Molecular Weight Compound as described herein; B is optional, and comprises a Spacer which may comprise one or more linkers Z; and Y is a PDGF Nucleic Acid Ligand.

"**Lipid Constructs**" for purposes of this invention, are structures containing lipids, phospholipids, or derivatives thereof comprising a variety of different structural arrangements which lipids are known to adopt in aqueous suspension. These structures include, but are not limited to, Lipid Bilayer Vesicles, micelles, Liposomes, emulsions, lipid ribbons or sheets, and  
5 may be complexed with a variety of drugs and components which are known to be pharmaceutically acceptable. In the preferred embodiment, the Lipid Construct is a Liposome. The preferred Liposome is unilamellar and has a relative size less than 200 nm. Common additional components in Lipid Constructs include cholesterol and alpha-tocopherol, among others. The Lipid Constructs may be used alone or in any combination which one skilled in  
10 the art would appreciate to provide the characteristics desired for a particular application. In addition, the technical aspects of Lipid Constructs and Liposome formation are well known in the art and any of the methods commonly practiced in the field may be used for the present invention.

"**Nucleic Acid Ligand**" as used herein is a non-naturally occurring Nucleic Acid  
15 having a desirable action on a Target. The Target of the present invention is PDGF, hence the term PDGF Nucleic Acid Ligand. A desirable action includes, but is not limited to, binding of the Target, catalytically changing the Target, reacting with the Target in a way which modifies/alters the Target or the functional activity of the Target, covalently attaching to the Target as in a suicide inhibitor, or facilitating the reaction between the Target and another  
20 molecule. In the preferred embodiment, the action is specific binding affinity for PDGF, wherein the Nucleic Acid Ligand is not a Nucleic Acid having the known physiological function of being bound by PDGF.

In preferred embodiments of the invention, the PDGF Nucleic Acid Ligand of the Complexes and Lipid Constructs of the invention are identified by the SELEX methodology.  
25 PDGF Nucleic Acid Ligands are identified from a Candidate Mixture of Nucleic Acids, said Nucleic Acid being a ligand of PDGF, by the method comprising a) contacting the Candidate Mixture with PDGF, wherein Nucleic Acids having an increased affinity to PDGF relative to the Candidate Mixture may be partitioned from the remainder of the Candidate Mixture; b) partitioning the increased affinity Nucleic Acids from the remainder of the Candidate Mixture;  
30 and c) amplifying the increased affinity Nucleic Acids to yield a ligand-enriched mixture of Nucleic Acids (see United States Patent Application Serial No. 08/479,725, filed June 7, 1995, entitled "High Affinity PDGF Nucleic Acid Ligands," now United States Patent No.

5,674,685, United States Patent Application Serial No. 08/479,783, filed June 7, 1995, entitled "High Affinity PDGF Nucleic Acid Ligands," now United States Patent No. 5,668,264, and United States Patent Application Serial No. 08/618,693, filed March 20, 1996, entitled "High Affinity PDGF Ligands," now United States Patent No. 5,723,564 which are hereby

5 incorporated by reference herein).

In certain embodiments, portions of the PDGF Nucleic Acid Ligand (Y) may not be necessary to maintain binding and certain portions of the contiguous PDGF Nucleic Acid Ligand can be replaced with a Spacer or Linker. In these embodiments, for example, Y can be represented as Y-B'-Y'-B"-Y", wherein Y, Y' and Y" are parts of a PDGF Nucleic Acid Ligand or segments of different PDGF Nucleic Acid Ligands and B' and/or B" are Spacers or Linker molecules that replace certain nucleic acid features of the original PDGF Nucleic Acid Ligand. When B' and B" are present and Y, Y', and Y" are parts of one PDGF Nucleic Acid Ligand, a tertiary structure is formed that binds to PDGF. When B' and B" are not present, Y, Y', and Y" represent one contiguous PDGF Nucleic Acid Ligand. PDGF Nucleic Acid Ligands modified in such a manner are included in this definition.

"Candidate Mixture" is a mixture of Nucleic Acids of differing sequence from which to select a desired ligand. The source of a Candidate Mixture can be from naturally-occurring Nucleic Acids or fragments thereof, chemically synthesized Nucleic Acids, enzymatically synthesized Nucleic Acids or Nucleic Acids made by a combination of the foregoing techniques. In a preferred embodiment, each Nucleic Acid has fixed sequences surrounding a randomized region to facilitate the amplification process.

"Nucleic Acid" means either DNA, RNA, single-stranded or double-stranded and any chemical modifications thereof. Modifications include, but are not limited to, those which provide other chemical groups that incorporate additional charge, polarizability, hydrogen bonding, electrostatic interaction, and fluxionality to the Nucleic Acid Ligand bases or to the Nucleic Acid Ligand as a whole. Such modifications include, but are not limited to, 2'-position sugar modifications, 5-position pyrimidine modifications, 8-position purine modifications, modifications at exocyclic amines, substitution of 4-thiouridine, substitution of 5-bromo or 5-iodo-uracil, backbone modifications such as internucleoside phosphorothioate linkages, methylations, unusual base-pairing combinations such as the isobases isocytidine and isoguanidine and the like. Modifications can also include 3' and 5' modifications such as capping.

"Non-Immunogenic, High Molecular Weight Compound" is a compound between approximately 1000 Da to 1,000,000 Da, more preferably approximately 1000 Da to 500,000 Da, and most preferably approximately 1000 Da to 200,000 Da, that typically does not generate an immunogenic response. For the purposes of this invention, an immunogenic response is one that causes the organism to make antibody proteins. Examples of Non-Immunogenic, High Molecular Weight Compounds include Polyalkylene Glycol and polyethylene glycol. In one preferred embodiment of the invention, the Non-Immunogenic, High Molecular Weight Compound covalently linked to the PDGF Nucleic Acid Ligand is a polyalkylene glycol and has the structure  $R(O(CH_2)_x)_nO^-$ , where R is independently selected from the group consisting of H and  $CH_3$ ,  $x=2-5$ , and  $n \approx MW \text{ of the Polyalkylene Glycol} / (16 + 14x)$ . In the preferred embodiment of the present invention, the molecular weight is about between 10-80 kDa. In the most preferred embodiment, the molecular weight of the polyalkylene glycol is about between 20-45 kDa. In the most preferred embodiment,  $x=2$  and  $n=9 \times 10^2$ . There can be one or more Polyalkylene Glycols attached to the same PDGF Nucleic Acid Ligand, with the sum of the molecular weights preferably being between 10-80 kDa, more preferably 20-45 kDa.

In certain embodiments, the Non-Immunogenic, High Molecular Weight Compound can also be a Nucleic Acid Ligand.

"Lipid Bilayer Vesicles" are closed, fluid-filled microscopic spheres which are formed principally from individual molecules having polar (hydrophilic) and non-polar (lipophilic) portions. The hydrophilic portions may comprise phosphato, glycerylphosphato, carboxy, sulfato, amino, hydroxy, choline and other polar groups. Examples of non-polar groups are saturated or unsaturated hydrocarbons such as alkyl, alkenyl or other lipid groups. Sterols (e.g., cholesterol) and other pharmaceutically acceptable components (including anti-oxidants like alpha-tocopherol) may also be included to improve vesicle stability or confer other desirable characteristics.

"Liposomes" are a subset of Lipid Bilayer Vesicles and are comprised principally of phospholipid molecules which contain two hydrophobic tails consisting of long fatty acid chains. Upon exposure to water, these molecules spontaneously align to form a bilayer membrane with the lipophilic ends of the molecules in each layer associated in the center of the membrane and the opposing polar ends forming the respective inner and outer surface of the bilayer membrane. Thus, each side of the membrane presents a hydrophilic surface while

the interior of the membrane comprises a lipophilic medium. These membranes when formed are generally arranged in a system of concentric closed membranes separated by interlamellar aqueous phases, in a manner not dissimilar to the layers of an onion, around an internal aqueous space. These multilamellar vesicles (MLV) can be converted into unilamellar vesicles (UV), with the application of a shearing force.

"**Cationic Liposome**" is a Liposome that contains lipid components that have an overall positive charge at physiological pH.

"**SELEX**" methodology involves the combination of selection of Nucleic Acid Ligands which interact with a Target in a desirable manner, for example binding to a protein, with amplification of those selected Nucleic Acids. Iterative cycling of the selection/amplification steps allows selection of one or a small number of Nucleic Acids which interact most strongly with the Target from a pool which contains a very large number of Nucleic Acids. Cycling of the selection/amplification procedure is continued until a selected goal is achieved. The SELEX methodology is described in the SELEX Patent Applications.

"**Target**" means any compound or molecule of interest for which a ligand is desired. A Target can be a protein (such as PDGF, thrombin, and selectin), peptide, carbohydrate, polysaccharide, glycoprotein, hormone, receptor, antigen, antibody, virus, substrate, metabolite, transition state analog, cofactor, inhibitor, drug, dye, nutrient, growth factor, etc. without limitation. The principal Target of the subject invention is PDGF.

"**Improved Pharmacokinetic Properties**" means that the PDGF Nucleic Acid Ligand covalently linked to a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound or in association with a Lipid Construct shows a longer circulation half-life *in vivo* relative to the same PDGF Nucleic Acid Ligand not in association with a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound or in association with a Lipid Construct.

"**Linker**" is a molecular entity that connects two or more molecular entities through Covalent Bond or Non-Covalent Interactions, and can allow spatial separation of the molecular entities in a manner that preserves the functional properties of one or more of the molecular entities. A linker can also be known as a Spacer. Examples of Linkers, include but are not limited to, the structures shown in Figures 9C-9E and the PEG spacer shown in Figure 9A.



In the preferred embodiment, the linker B' and B" are pentaethylene glycols.

**"Therapeutic"** as used herein, includes treatment and/or prophylaxis. When used, Therapeutic refers to humans and other animals.

This invention includes ssDNA and RNA ligands to PDGF. This invention further  
5 includes the specific ssDNA and RNA ligands to PDGF shown in Tables 2-3, 6-7, and 9 and  
Figures 1-2A, 2B and 2C, 8A and 8B (SEQ ID NOS:4-35, 39-87, 97-144, 148-149). More  
specifically, this invention includes nucleic acid sequences that are substantially homologous  
to and that have substantially the same ability to bind PDGF as the specific nucleic acid  
ligands shown in Tables 2-3, 6-7, and 9 and Figures 1-2A, 2B and 2C, 8A and 8B (SEQ ID  
10 NOS:4-35, 39-87, 97-144, 148-149). By substantially homologous it is meant a degree of  
primary sequence homology in excess of 70%, most preferably in excess of 80%, and even  
more preferably in excess of 90%, 95%, or 99%. The percentage of homology as described  
herein is calculated as the percentage of nucleotides found in the smaller of the two sequences  
which align with identical nucleotide residues in the sequence being compared when 1 gap in  
15 a length of 10 nucleotides may be introduced to assist in that alignment. Substantially the  
same ability to bind PDGF means that the affinity is within one or two orders of magnitude of  
the affinity of the ligands described herein. It is well within the skill of those of ordinary skill  
in the art to determine whether a given sequence – substantially homologous to those  
specifically described herein – has the same ability to bind PDGF.

20 A review of the sequence homologies of the nucleic acid ligands of PDGF shown in  
Tables 2-3, 6-7, and 9 and Figures 1-2A, 2 B and 2C, 8A and 8B (SEQ ID NOS:4-35, 39-87,  
97-144, 148-149) shows that sequences with little or no primary homology may have  
substantially the same ability to bind PDGF. For these reasons, this invention also includes  
Nucleic Acid Ligands that have substantially the same postulated structure or structural motifs  
25 and ability to bind PDGF as the nucleic acid ligands shown in Tables 2-3, 6-7, and 9 and  
Figures 1-2A, 2B and 2C, 8A and 8B (SEQ ID NOS:4-35, 39-87, 97-144, 148-149).  
Substantially the same structure or structural motifs can be postulated by sequence alignment  
using the Zukerfold program (see Zuker (1989) Science 244:48-52). As would be known in  
the art, other computer programs can be used for predicting secondary structure and structural  
30 motifs. Substantially the same structure or structural motif of Nucleic Acid Ligands in  
solution or as a bound structure can also be postulated using NMR or other techniques as  
would be known in the art.

Further included in this invention is a method for preparing a Complex comprised of a PDGF Nucleic Acid Ligand and a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound by the method comprising identifying a Nucleic Acid Ligand from a Candidate Mixture of Nucleic Acids where the Nucleic Acid is a ligand of PDGF by the method of (a) contacting the Candidate Mixture of Nucleic Acids with PDGF, (b) partitioning between members of said Candidate Mixture on the basis of affinity to PDGF, and c) amplifying the selected molecules to yield a mixture of Nucleic Acids enriched for Nucleic Acid sequences with a relatively higher affinity for binding to PDGF, and covalently linking said identified PDGF Nucleic Acid Ligand with a Non-Immunogenic, High Molecular Weight Compound or a Lipophilic Compound.

It is a further object of the present invention to provide Complexes comprising one or more PDGF Nucleic Acid Ligands covalently linked to a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound. Such Complexes have one or more of the following advantages over a PDGF Nucleic Acid Ligand not in association with a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound: 1) Improved Pharmacokinetic Properties, and 2) improved capacity for intracellular delivery, or 3) improved capacity for targeting. Complexes further associated with a Lipid Construct have the same advantages.

The Complexes or the Lipid Constructs comprising the PDGF Nucleic Acid Ligand or Complexes may benefit from one, two, or three of these advantages. For example, a Lipid Construct of the present invention may be comprised of a) a Liposome, b) a drug that is encapsulated within the interior of the Liposome, and c) a Complex comprised of a PDGF Nucleic Acid Ligand and Lipophilic Compound, wherein the PDGF Nucleic Acid Ligand component of the Complex is associated with and projecting from the exterior of the Lipid Construct. In such a case, the Lipid Construct comprising a Complex will 1) have Improved Pharmacokinetic Properties, 2) have enhanced capacity for intracellular delivery of the encapsulated drug, and 3) be specifically targeted to the preselected location *in vivo* that is expressing PDGF by the exteriorly associated PDGF Nucleic Acid Ligand.

In another embodiment, this invention provides a method for improving the pharmacokinetic properties of a PDGF Nucleic Acid Ligand by covalently linking the PDGF Nucleic Acid Ligand with a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound to form a Complex and administering the Complex to a patient. The

invention further relates to a method for improving the pharmacokinetic properties of a PDGF Nucleic Acid Ligand by further associating the Complex with a Lipid Construct.

In another embodiment, the Complex of the present invention is comprised of a PDGF Nucleic Acid Ligand covalently attached to a Lipophilic Compound, such as a glycerolipid, or a Non-Immunogenic, High Molecular Weight Compound, such as polyalkylene glycol or polyethylene glycol (PEG). In these cases, the pharmacokinetic properties of the Complex will be enhanced relative to the PDGF Nucleic Acid Ligand alone. In another embodiment, the pharmacokinetic properties of the PDGF Nucleic Acid Ligand is enhanced relative to the PDGF Nucleic Acid Ligand alone when the PDGF Nucleic Acid Ligand is covalently attached to a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound and is further associated with a Lipid Construct or the PDGF Nucleic Acid Ligand is encapsulated within a Lipid Construct.

In embodiments where there are multiple PDGF Nucleic Acid Ligands, there is an increase in avidity due to multiple binding interactions with PDGF. Furthermore, in embodiments where the Complex is comprised of multiple PDGF Nucleic Acid Ligands, the pharmacokinetic properties of the Complex will be improved relative to one PDGF Nucleic Acid Ligand alone. In embodiments where a Lipid Construct comprises multiple Nucleic Acid Ligands or Complexes, the Pharmacokinetic Properties of the PDGF Nucleic Acid Ligand may be improved relative to Lipid Constructs in which there is only one Nucleic Acid Ligand or Complex.

In certain embodiments of the invention, the Complex of the present invention is comprised of a PDGF Nucleic Acid Ligand attached to one (dimeric) or more (multimeric) other Nucleic Acid Ligands. The Nucleic Acid Ligand can be to PDGF or a different Target. In embodiments where there are multiple PDGF Nucleic Acid Ligands, there is an increase in avidity due to multiple binding interactions with PDGF. Furthermore, in embodiments of the invention where the Complex is comprised of a PDGF Nucleic Acid Ligand attached to one or more other PDGF Nucleic Acid Ligands, the pharmacokinetic properties of the Complex will be improved relative to one PDGF Nucleic Acid Ligand alone.

The Non-Immunogenic, High Molecular Weight compound or Lipophilic Compound may be covalently bound to a variety of positions on the PDGF Nucleic Acid Ligand, such as to an exocyclic amino group on the base, the 5-position of a pyrimidine nucleotide, the 8-

position of a purine nucleotide, the hydroxyl group of the phosphate, or a hydroxyl group or other group at the 5' or 3' terminus of the PDGF Nucleic Acid Ligand. In embodiments where the Non-Immunogenic, High Molecular Weight Compound is polyalkylene glycol or polyethylene glycol, preferably it is bonded to the 5' or 3' hydroxyl of the phosphate group thereof. In the most preferred embodiment, the Non-Immunogenic, High Molecular Weight Compound is bonded to the 5' hydroxyl of the phosphate group of the Nucleic Acid Ligand. Attachment of the Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound to the PDGF Nucleic Acid Ligand can be done directly or with the utilization of Linkers or Spacers. In embodiments where the Lipid Construct comprises a Complex, or where the PDGF Nucleic Acid Ligands are encapsulated within the Liposome, a Non-Covalent Interaction between the PDGF Nucleic Acid Ligand or the Complex and the Lipid Construct is preferred.

One problem encountered in the therapeutic use of Nucleic Acids is that oligonucleotides in their phosphodiester form may be quickly degraded in body fluids by intracellular and extracellular enzymes such as endonucleases and exonucleases before the desired effect is manifest. Certain chemical modifications of the PDGF Nucleic Acid Ligand can be made to increase the *in vivo* stability of the PDGF Nucleic Acid Ligand or to enhance or to mediate the delivery of the PDGF Nucleic Acid Ligand. Modifications of the PDGF Nucleic Acid Ligands contemplated in this invention include, but are not limited to, those which provide other chemical groups that incorporate additional charge, polarizability, hydrophobicity, hydrogen bonding, electrostatic interaction, and fluxionality to the PDGF Nucleic Acid Ligand bases or to the PDGF Nucleic Acid Ligand as a whole. Such modifications include, but are not limited to, 2'-position sugar modifications, 5-position pyrimidine modifications, 8-position purine modifications, modifications at exocyclic amines, substitution of 4-thiouridine, substitution of 5-bromo or 5-iodo-uracil; backbone modifications, phosphorothioate or alkyl phosphate modifications, methylations, unusual base-pairing combinations such as the isobases isocytidine and isoguanidine and the like. Modifications can also include 3' and 5' modifications such as capping.

Where the Nucleic Acid Ligands are derived by the SELEX method, the modifications can be pre- or post- SELEX modifications. Pre-SELEX modifications yield PDGF Nucleic Acid Ligands with both specificity for PDGF and improved *in vivo* stability. Post-SELEX modifications made to 2'-OH Nucleic Acid Ligands can result in improved *in vivo* stability

without adversely affecting the binding capacity of the Nucleic Acid Ligands. The preferred modifications of the PDGF Nucleic Acid Ligands of the subject invention are 5' and 3' phosphorothioate capping and 3'3' inverted phosphodiester linkage at the 3' end. In the most preferred embodiment, the preferred modification of the PDGF Nucleic Acid Ligand is 3'3' inverted phosphodiester linkage at the 3' end. Additional 2' fluoro (2'-F), 2' amino (2'-NH<sub>2</sub>) and 2' OMethyl (2'-OMe) modification of all or some of the nucleotides is preferred. In the most preferred embodiment, the preferred modification is 2'-OMe and 2'-F modification of some of the nucleotides. Additionally, the PDGF Nucleic Acid Ligand can be post-SELEX modified to substitute Linkers or Spacers such as hexaethylene glycol Spacers for certain portions.

In another aspect of the present invention, the covalent linking of the PDGF Nucleic Acid Ligand with a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound results in Improved Pharmacokinetic Properties (i.e., slower clearance rate) relative to the PDGF Nucleic Acid Ligand not in association with a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound.

In another aspect of the present invention, the Complex comprising a PDGF Nucleic Acid Ligand and Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound can be further associated with a Lipid Construct. This association may result in Improved Pharmacokinetic Properties relative to the PDGF Nucleic Acid Ligand or Complex not in association with a Lipid Construct. The PDGF Nucleic Acid Ligand or Complex can be associated with the Lipid Construct through covalent or Non-Covalent Interactions. In another aspect, the PDGF Nucleic Acid Ligand can be associated with the Lipid Construct through Covalent or Non-Covalent Interactions. In a preferred embodiment, the association is through Non-Covalent Interactions. In a preferred embodiment, the Lipid Construct is a Lipid Bilayer Vesicle. In the most preferred embodiment, the Lipid Construct is a Liposome.

Liposomes for use in the present invention can be prepared by any of the various techniques presently known in the art or subsequently developed. Typically, they are prepared from a phospholipid, for example, distearoyl phosphatidylcholine, and may include other materials such as neutral lipids, for example, cholesterol, and also surface modifiers such as positively charged (e.g., sterylamine or aminomannose or aminomannitol derivatives of cholesterol) or negatively charged (e.g., diacetyl phosphate, phosphatidyl glycerol) compounds. Multilamellar Liposomes can be formed by conventional techniques, that is, by

depositing a selected lipid on the inside wall of a suitable container or vessel by dissolving the lipid in an appropriate solvent, and then evaporating the solvent to leave a thin film on the inside of the vessel or by spray drying. An aqueous phase is then added to the vessel with a swirling or vortexing motion which results in the formation of MLVs. UVs can then be  
5 formed by homogenization, sonication or extrusion (through filters) of MLV's. In addition, UVs can be formed by detergent removal techniques.

In certain embodiments of this invention, the Lipid Construct comprises a targeting PDGF Nucleic Acid Ligand(s) associated with the surface of the Lipid Construct and an encapsulated therapeutic or diagnostic agent. Preferably the Lipid Construct is a Liposome.  
10 Preformed Liposomes can be modified to associate with the PDGF Nucleic Acid Ligands. For example, a Cationic Liposome associates through electrostatic interactions with the PDGF Nucleic Acid Ligand. A PDGF Nucleic Acid Ligand covalently linked to a Lipophilic Compound, such as a glycerolipid, can be added to preformed Liposomes whereby the glycerolipid, phospholipid, or glycerol amide lipid becomes associated with the  
15 liposomal membrane. Alternatively, the PDGF Nucleic Acid Ligand can be associated with the Liposome during the formulation of the Liposome.

It is well known in the art that Liposomes are advantageous for encapsulating or incorporating a wide variety of therapeutic and diagnostic agents. Any variety of compounds can be enclosed in the internal aqueous compartment of the Liposomes.  
20 Illustrative therapeutic agents include antibiotics, antiviral nucleosides, antifungal nucleosides, metabolic regulators, immune modulators, chemotherapeutic drugs, toxin antidotes, DNA, RNA, antisense oligonucleotides, etc. By the same token, the Lipid Bilayer Vesicles may be loaded with a diagnostic radionuclide (e.g., indium 111, iodine 131, yttrium 90, phosphorous 32, or gadolinium) and fluorescent materials or other  
25 materials that are detectable in *in vitro* and *in vivo* applications. It is to be understood that the therapeutic or diagnostic agent can be encapsulated by the Liposome walls in the aqueous interior. Alternatively, the carried agent can be a part of, that is, dispersed or dissolved in the vesicle wall-forming materials.

During Liposome formation, water soluble carrier agents may be encapsulated in the  
30 aqueous interior by including them in the hydrating solution, and lipophilic molecules incorporated into the lipid bilayer by inclusion in the lipid formulation. In the case of certain molecules (e.g., cationic or anionic lipophilic drugs), loading of the drug into

preformed Liposomes may be accomplished, for example, by the methods described in U.S. Patent No. 4,946,683, the disclosure of which is incorporated herein by reference.

Following drug encapsulation, the Liposomes are processed to remove unencapsulated drug through processes such as gel chromatography or ultrafiltration. The Liposomes are then  
5 typically sterile filtered to remove any microorganisms which may be present in the suspension. Microorganisms may also be removed through aseptic processing.

If one wishes to encapsulate large hydrophilic molecules with Liposomes, larger unilamellar vesicles can be formed by methods such as the reverse-phase evaporation (REV) or solvent infusion methods. Other standard methods for the formation of Liposomes are  
10 known in the art, for example, methods for the commercial production of Liposomes include the homogenization procedure described in U.S. Patent No. 4,753,788 and the thin-film evaporation method described in U.S. Patent No. 4,935,171, which are incorporated herein by reference.

It is to be understood that the therapeutic or diagnostic agent can also be associated  
15 with the surface of the Lipid Bilayer Vesicle. For example, a drug can be attached to a phospholipid or glyceride (a prodrug). The phospholipid or glyceride portion of the prodrug can be incorporated into the lipid bilayer of the Liposome by inclusion in the lipid formulation or loading into preformed Liposomes (see U.S. Patent Nos 5,194,654 and 5,223,263, which are incorporated by reference herein).

20 It is readily apparent to one skilled in the art that the particular Liposome preparation method will depend on the intended use and the type of lipids used to form the bilayer membrane.

Lee and Low (1994, JBC 269:3198-3204) and DeFrees *et al.* (1996, J. Am. Chem. Soc. 118:6101-6104) first showed that co-formulation of ligand-PEG-lipid with lipid  
25 components gave liposomes with both inward and outward facing orientations of the PEG-ligand. Passive anchoring was outlined by Zalipsky *et al.* (1997, Bioconj. Chem. 8:111-118) as a method for anchoring oligopeptide and oligosaccharide ligands exclusively to the external surface of liposomes. The central concept presented in their work is that oligo-PEG-lipid conjugates can be prepared and then formulated into pre-formed liposomes via  
30 spontaneous incorporation ("anchoring") of the lipid tail into the existing lipid bilayer. The lipid group undergoes this insertion in order to reach a lower free energy state via the removal of its hydrophobic lipid anchor from aqueous solution and its subsequent

positioning in the hydrophobic lipid bilayer. The key advantage to such a system is that the oligo-lipid is anchored exclusively to the exterior of the lipid bilayer. Thus, no oligo-lipids are wasted by being unavailable for interactions with their biological targets by being in an inward-facing orientation.

5           The efficiency of delivery of a PDGF Nucleic Acid Ligand to cells may be optimized by using lipid formulations and conditions known to enhance fusion of Liposomes with cellular membranes. For example, certain negatively charged lipids such as phosphatidylglycerol and phosphatidylserine promote fusion, especially in the presence of other fusogens (e.g., multivalent cations like  $\text{Ca}^{2+}$ , free fatty acids, viral fusion proteins,  
10 short chain PEG, lysolecithin, detergents and surfactants). Phosphatidylethanolamine may also be included in the Liposome formulation to increase membrane fusion and, concomitantly, enhance cellular delivery. In addition, free fatty acids and derivatives thereof, containing, for example, carboxylate moieties, may be used to prepare pH-sensitive Liposomes which are negatively charged at higher pH and neutral or protonated at lower  
15 pH. Such pH-sensitive Liposomes are known to possess a greater tendency to fuse.

In the preferred embodiment, the PDGF Nucleic Acid Ligands of the present invention are derived from the SELEX methodology. SELEX is described in United States Patent Application Serial No. 07/536,428, entitled "Systematic Evolution of Ligands by Exponential Enrichment," now abandoned, United States Patent Application Serial No. 07/714,131, filed  
20 June 10, 1991, entitled "Nucleic Acid Ligands," now United States Patent No. 5,475,096, United States Patent Application Serial No. 07/931,473, filed August 17, 1992, entitled "Methods of Identifying Nucleic Acid Ligands," now United States Patent No. 5,270,163 (see also WO 91/19813). These applications, each specifically incorporated herein by reference, are collectively called the SELEX Patent Applications.

25           The SELEX process provides a class of products which are Nucleic Acid molecules, each having a unique sequence, and each of which has the property of binding specifically to a desired Target compound or molecule. Target molecules are preferably proteins, but can also include among others carbohydrates, peptidoglycans and a variety of small molecules. SELEX methodology can also be used to target biological structures, such as cell surfaces or  
30 viruses, through specific interaction with a molecule that is an integral part of that biological structure.



In its most basic form, the SELEX process may be defined by the following series of steps:

- 1) A Candidate Mixture of Nucleic Acids of differing sequence is prepared. The Candidate Mixture generally includes regions of fixed sequences (i.e., each of the members of  
5 the Candidate Mixture contains the same sequences in the same location) and regions of randomized sequences. The fixed sequence regions are selected either: (a) to assist in the amplification steps described below, (b) to mimic a sequence known to bind to the Target, or (c) to enhance the concentration of a given structural arrangement of the Nucleic Acids in the Candidate Mixture. The randomized sequences can be totally randomized (i.e., the probability  
10 of finding a base at any position being one in four) or only partially randomized (e.g., the probability of finding a base at any location can be selected at any level between 0 and 100 percent).
- 2) The Candidate Mixture is contacted with the selected Target under conditions favorable for binding between the Target and members of the Candidate Mixture. Under these  
15 circumstances, the interaction between the Target and the Nucleic Acids of the Candidate Mixture can be considered as forming Nucleic Acid-target pairs between the Target and those Nucleic Acids having the strongest affinity for the Target.
- 3) The Nucleic Acids with the highest affinity for the target are partitioned from those Nucleic Acids with lesser affinity to the target. Because only an extremely small number of  
20 sequences (and possibly only one molecule of Nucleic Acid) corresponding to the highest affinity Nucleic Acids exist in the Candidate Mixture, it is generally desirable to set the partitioning criteria so that a significant amount of the Nucleic Acids in the Candidate Mixture (approximately 5-50%) are retained during partitioning.
- 4) Those Nucleic Acids selected during partitioning as having the relatively higher  
25 affinity for the target are then amplified to create a new Candidate Mixture that is enriched in Nucleic Acids having a relatively higher affinity for the target.
- 5) By repeating the partitioning and amplifying steps above, the newly formed Candidate Mixture contains fewer and fewer unique sequences, and the average degree of affinity of the Nucleic Acids to the target will generally increase. Taken to its extreme, the  
30 SELEX process will yield a Candidate Mixture containing one or a small number of unique Nucleic Acids representing those Nucleic Acids from the original Candidate Mixture having the highest affinity to the target molecule.

The basic SELEX method has been modified to achieve a number of specific objectives. For example, United States Patent Application Serial No. 07/960,093, filed October 14, 1992, entitled "Method for Selecting Nucleic Acids on the Basis of Structure," describes the use of SELEX in conjunction with gel electrophoresis to select Nucleic Acid molecules with specific structural characteristics, such as bent DNA. United States Patent Application Serial No. 08/123,935, filed September 17, 1993, entitled "Photoselection of Nucleic Acid Ligands," describes a SELEX based method for selecting Nucleic Acid Ligands containing photoreactive groups capable of binding and/or photocrosslinking to and/or photoinactivating a target molecule. United States Patent Application Serial No. 08/134,028, filed October 7, 1993, entitled "High-Affinity Nucleic Acid Ligands That Discriminate Between Theophylline and Caffeine," abandoned in favor of United States Patent Application Serial No. 08/443,957, now United States Patent No. 5,580,737, describes a method for identifying highly specific Nucleic Acid Ligands able to discriminate between closely related molecules, termed Counter-SELEX. United States Patent Application Serial No. 08/143,564, filed October 25, 1993, entitled "Systematic Evolution of Ligands by Exponential Enrichment: Solution SELEX," abandoned in favor of United States Patent Application Serial No. 08/461,069, now United States Patent No. 5,567,588, describes a SELEX-based method which achieves highly efficient partitioning between oligonucleotides having high and low affinity for a target molecule. United States Patent Application Serial No. 07/964,624, filed October 21, 1992, entitled "Nucleic Acid Ligands to HIV-RT and HIV-1 Rev," abandoned in favor of United States Patent Application No. 08/462,260, now United States Patent No. 5,496,938, describes methods for obtaining improved Nucleic Acid Ligands after SELEX has been performed. United States Patent Application Serial No. 08/400,440, filed March 8, 1995, entitled "Systematic Evolution of Ligands by Exponential Enrichment: Chemi-SELEX," now United States Patent No. 5,705,337, describes methods for covalently linking a ligand to its target.

The SELEX method encompasses the identification of high-affinity Nucleic Acid Ligands containing modified nucleotides conferring improved characteristics on the ligand, such as improved *in vivo* stability or improved delivery characteristics. Examples of such modifications include chemical substitutions at the ribose and/or phosphate and/or base positions. SELEX-identified Nucleic Acid Ligands containing modified nucleotides are described in United States Patent Application Serial No. 08/117,991, filed September 8, 1993,

entitled "High Affinity Nucleic Acid Ligands Containing Modified Nucleotides," abandoned in favor of United States Patent Application Serial No. 08/430,709, now United States Patent No. 5,660,985, that describes oligonucleotides containing nucleotide derivatives chemically modified at the 5- and 2'-positions of pyrimidines. United States Patent Application Serial No. 08/134,028, *supra*, describes highly specific Nucleic Acid Ligands containing one or more nucleotides modified with 2'-amino (2'-NH<sub>2</sub>), 2'-fluoro (2'-F), and/or 2'-O-methyl (2'-OMe). United States Patent Application Serial No. 08/264,029, filed June 22, 1994, entitled "Novel Method of Preparation of Known and Novel 2'-Modified Nucleosides by Intramolecular Nucleophilic Displacement," describes oligonucleotides containing various 2'-modified pyrimidines.

The SELEX method encompasses combining selected oligonucleotides with other selected oligonucleotides and non-oligonucleotide functional units as described in United States Patent Application Serial No. 08/284,063, filed August 2, 1994, entitled "Systematic Evolution of Ligands by Exponential Enrichment: Chimeric SELEX," now United States Patent No. 5,637,459, and United States Patent Application Serial No. 08/234,997, filed April 28, 1994, entitled "Systematic Evolution of Ligands by Exponential Enrichment: Blended SELEX," now United States Patent No. 5,683,867, respectively. These applications allow the combination of the broad array of shapes and other properties, and the efficient amplification and replication properties, of oligonucleotides with the desirable properties of other molecules.

The SELEX method further encompasses combining selected Nucleic Acid Ligands with Lipophilic Compounds or Non-Immunogenic, High Molecular Weight Compounds in a diagnostic or therapeutic Complex as described in United States Patent Application Serial No. 08/434,465, filed May 4, 1995, entitled "Nucleic Acid Ligand Complexes." The SELEX method further encompasses combining selected VEGF Nucleic Acid Ligands with lipophilic compounds, such as diacyl glycerol or dialkyl glycerol, as described in United States Patent Application Serial No. 08/739,109, filed October 25, 1996, entitled "Vascular Endothelial Growth Factor (VEGF) Nucleic Acid Ligand Complexes." VEGF Nucleic Acid Ligands that are associated with a High Molecular Weight, Non-Immunogenic Compound, such as polyethylene glycol, or a Lipophilic Compound, such as glycerolipid, phospholipid, or glycerol amide lipid, in a diagnostic or therapeutic complex are described in United States Patent Application Serial No. 08/897,351, filed July 21, 1997, entitled "Vascular Endothelial Growth Factor (VEGF) Nucleic Acid Ligand Complexes." Each of the above described patent

applications which describe modifications of the basic SELEX procedure are specifically incorporated by reference herein in their entirety.

SELEX identifies Nucleic Acid Ligands that are able to bind targets with high affinity and with outstanding specificity, which represents a singular achievement that is  
5   unprecedented in the field of Nucleic Acids research. These characteristics are, of course, the desired properties one skilled in the art would seek in a therapeutic or diagnostic ligand.

In order to produce Nucleic Acid Ligands desirable for use as a pharmaceutical, it is preferred that the Nucleic Acid Ligand (1) binds to the target in a manner capable of achieving the desired effect on the target; (2) be as small as possible to obtain the desired effect; (3) be as  
10   stable as possible; and (4) be a specific ligand to the chosen target. In most situations, it is preferred that the Nucleic Acid Ligand has the highest possible affinity to the target. Additionally, Nucleic Acid Ligands can have facilitating properties.

In commonly assigned United States Patent Application Serial No. 07/964,624, filed October 21, 1992, now United States Patent No. 5,496,938 ('938), methods are described for  
15   obtaining improved Nucleic Acid Ligands after SELEX has been performed. The '938 patent, entitled "Nucleic Acid Ligands to HIV-RT and HIV-1 Rev," is specifically incorporated herein by reference.

The SELEX process has been used to identify a group of high affinity RNA Ligands to PDGF from random ssDNA libraries and 2'-fluoro-2'-deoxypyrimidine RNA ligands from  
20   random ssDNA libraries (United States Patent Application Serial No. 08/618,693, filed March 20, 1996, entitled "High-Affinity PDGF Nucleic Acid Ligands," which is a Continuation-in-Part Application of United States Patent Application Serial No. 08/479,783, filed June 7, 1995, entitled "High-Affinity PDGF Nucleic Acid Ligands," and United States Patent Application Serial No. 08/479,725, filed June 7, 1995, entitled "High Affinity PDGF Nucleic  
25   Acid Ligands," each of which is incorporated herein by reference; see also Green *et al.* (1995) Chemistry and Biology 2:683-695).

In embodiments where the PDGF Nucleic Acid Ligand(s) can serve in a targeting capacity, the PDGF Nucleic Acid Ligands adopt a three dimensional structure that must be retained in order for the PDGF Nucleic Acid Ligand to be able to bind its target. In  
30   embodiments where the Lipid Construct comprises a Complex and the PDGF Nucleic Acid Ligand of the Complex is projecting from the surface of the Lipid Construct, the PDGF Nucleic Acid Ligand must be properly oriented with respect to the surface of the Lipid

Construct so that its target binding capacity is not compromised. This can be accomplished by attaching the PDGF Nucleic Acid Ligand at a position that is distant from the binding portion of the PDGF Nucleic Acid Ligand. The three dimensional structure and proper orientation can also be preserved by use of a Linker or Spacer as described *supra*.

5       Any variety of therapeutic or diagnostic agents can be attached to the Complex for targeted delivery by the Complex. In addition, any variety of therapeutic or diagnostic agents can be attached encapsulated, or incorporated into the Lipid Construct as discussed *supra* for targeted delivery by the Lipid Construct.

10       In embodiments where the Complex is comprised of a Lipophilic Compound and a PDGF Nucleic Acid Ligand in association with a Liposome, for example, the PDGF Nucleic Acid Ligand could target tumor cells expressing PDGF (e.g., in Kaposi's sarcoma) for delivery of an antitumor drug (e.g., daunorubicin) or imaging agent (e.g., radiolabels). It should be noted that cells and tissues surrounding the tumor may also express PDGF, and targeted delivery of an antitumor drug to these cells would also be effective.

15       In an alternative embodiment, the therapeutic or diagnostic agent to be delivered to the Target cell could be another Nucleic Acid Ligand.

20       It is further contemplated by this invention that the agent to be delivered can be incorporated into the Complex in such a way as to be associated with the outside surface of the Liposome (e.g., a prodrug, receptor antagonist, or radioactive substance for treatment or imaging). As with the PDGF Nucleic Acid Ligand, the agent can be associated through covalent or Non-Covalent Interactions. The Liposome would provide targeted delivery of the agent extracellularly, with the Liposome serving as a Linker.

25       In another embodiment, a Non-Immunogenic, High Molecular Weight Compound (e.g., PEG) can be attached to the Liposome to provide improved pharmacokinetic properties for the Complex. PDGF Nucleic Acid Ligands may be attached to the Liposome membrane or may be attached to a Non-Immunogenic, High Molecular Weight Compound which in turn is attached to the membrane. In this way, the Complex may be shielded from blood proteins and thus be made to circulate for extended periods of time while the PDGF Nucleic Acid Ligand is still sufficiently exposed to make contact with and bind to its Target.

30       In another embodiment of the present invention, more than one PDGF Nucleic Acid Ligand is attached to the surface of the same Liposome. This provides the possibility of

bringing the same PDGF molecules in close proximity to each other and can be used to generate specific interactions between the PDGF molecules.

In an alternative embodiment of the present invention, PDGF Nucleic Acid Ligands and a Nucleic Acid Ligand to a different Target can be attached to the surface of the same Liposome. This provides the possibility of bringing PDGF in close proximity to a different Target and can be used to generate specific interactions between PDGF and the other Target. In addition to using the Liposome as a way of bringing Targets in close proximity, agents could be encapsulated in the Liposome to increase the intensity of the interaction.

The Lipid Construct comprising a Complex allows for the possibility of multiple binding interactions to PDGF. This, of course, depends on the number of PDGF Nucleic Acid Ligands per Complex, and the number of Complexes per Lipid Construct, and mobility of the PDGF Nucleic Acid Ligands and receptors in their respective membranes. Since the effective binding constant may increase as the product of the binding constant for each site, there is a substantial advantage to having multiple binding interactions. In other words, by having many PDGF Nucleic Acid Ligands attached to the Lipid Construct, and therefore creating multivalency, the effective affinity (i.e., the avidity) of the multimeric Complex for its Target may become as good as the product of the binding constant for each site.

In certain embodiments of the invention, the Complex of the present invention is comprised of a PDGF Nucleic Acid Ligand attached to a Lipophilic Compound. In this case, the pharmacokinetic properties of the Complex will be improved relative to the PDGF Nucleic Acid Ligand alone. As discussed *supra*, the Lipophilic Compound may be covalently bound to the PDGF Nucleic Acid Ligand at numerous positions on the PDGF Nucleic Acid Ligand.

In another embodiment of the invention, the Lipid Construct comprises a PDGF Nucleic Acid Ligand or Complex. In this embodiment, the glycerolipid can assist in the incorporation of the PDGF Nucleic Acid Ligand into the Liposome due to the propensity for a glycerolipid to associate with other Lipophilic Compounds. The glycerolipid in association with a PDGF Nucleic Acid Ligand can be incorporated into the lipid bilayer of the Liposome by inclusion in the formulation or by loading into preformed Liposomes. The glycerolipid can associate with the membrane of the Liposome in such a way so as the PDGF Nucleic Acid Ligand is projecting into or out of the Liposome. In embodiments

where the PDGF Nucleic Acid Ligand is projecting out of the Complex, the PDGF Nucleic Acid Ligand can serve in a targeting capacity. It is to be understood that additional compounds can be associated with the Lipid Construct to further improve the pharmacokinetic properties of the Lipid Construct. For example, a PEG may be attached to the exterior-facing part of the membrane of the Lipid Construct.

In other embodiments, the Complex of the present invention is comprised of a PDGF Nucleic Acid Ligand covalently linked to a Non-Immunogenic, High Molecular Weight Compound such as polyalkylene glycol or PEG. In this embodiment, the pharmacokinetic properties of the Complex are improved relative to the PDGF Nucleic Acid Ligand alone. The polyalkylene glycol or PEG may be covalently bound to a variety of positions on the PDGF Nucleic Acid Ligand. In embodiments where polyalkylene glycol or PEG are used, it is preferred that the PDGF Nucleic Acid Ligand is bonded through the 5' hydroxyl group via a phosphodiester linkage.

In certain embodiments, a plurality of Nucleic Acid Ligands can be associated with a single Non-Immunogenic, High Molecular Weight Compound, such as polyalkylene glycol or PEG, or a Lipophilic Compound, such as a glycerolipid. The Nucleic Acid Ligands can all be to PDGF or PDGF and a different Target. In embodiments where there are multiple PDGF Nucleic Acid Ligands, there is an increase in avidity due to multiple binding interactions with PDGF. In yet further embodiments, a plurality of polyalkylene glycol, PEG, glycerol lipid molecules can be attached to each other. In these embodiments, one or more PDGF Nucleic Acid Ligands or Nucleic Acid Ligands to PDGF and other Targets can be associated with each polyalkylene glycol, PEG, or glycerol lipid. This also results in an increase in avidity of each Nucleic Acid Ligand to its Target. In embodiments where multiple PDGF Nucleic Acid Ligands are attached to polyalkylene glycol, PEG, or glycerol lipid, there is the possibility of bringing PDGF molecules in close proximity to each other in order to generate specific interactions between PDGF. Where multiple Nucleic Acid Ligands specific for PDGF and different Targets are attached to polyalkylene glycol, PEG, or glycerol lipid, there is the possibility of bringing PDGF and another Target in close proximity to each other in order to generate specific interactions between the PDGF and the other Target. In addition, in embodiments where there are Nucleic Acid Ligands to PDGF or Nucleic Acid Ligands to PDGF and different Targets associated with polyalkylene glycol, PEG, or glycerol lipid, a drug can also be associated with polyalkylene glycol, PEG,

or glycerol lipid. Thus the Complex would provide targeted delivery of the drug, with polyalkylene glycol, PEG, or glycerol lipid serving as a Linker.

PDGF Nucleic Acid Ligands selectively bind PDGF. Thus, a Complex comprising a PDGF Nucleic Acid Ligand and a Non-Immunogenic, High Molecular Weight Compound  
5 or Lipophilic Compound or a Lipid Construct comprising a PDGF Nucleic Acid Ligand or a Complex are useful as pharmaceuticals or diagnostic agents. The PDGF Nucleic Acid Ligand-containing Complexes and Lipid Constructs can be used to treat, inhibit, prevent or diagnose any disease state that involves inappropriate PDGF production, for example, cancer, angiogenesis, restenosis, and fibrosis. PDGF is produced and secreted in varying  
10 amounts by many tumor cells. Thus, the present invention, includes methods of treating, inhibiting, preventing, or diagnosing cancer by administration of a Complex comprising a PDGF Nucleic Acid Ligand and a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound, a Lipid Construct comprising a Complex, or a PDGF Nucleic Acid Ligand in association with a Lipid Construct without being part of the Complex.

15 Angiogenesis rarely occurs in healthy adults, except during the menstrual cycle and wound healing. Angiogenesis is a central feature, however, of various disease states, including, but not limited to cancer, diabetic retinopathy, macular degeneration, psoriasis and rheumatoid arthritis. The present invention, therefore, includes methods of treating, inhibiting, preventing, or diagnosing angiogenesis by administration of a Complex  
20 comprising PDGF Nucleic Acid Ligand and a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound, a Lipid Construct comprising PDGF Nucleic Acid Ligand or a Complex comprising a PDGF Nucleic Acid Ligand and a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound.

PDGF is also produced in fibrosis in organs, such as lung, bone marrow and kidney.  
25 Fibrosis can also be associated with radiation treatments. The present invention, therefore, includes methods of treating, inhibiting, preventing or diagnosing lung, bone marrow, kidney and radiation treatment-associated fibrosis by administration of a Complex comprising PDGF Nucleic Acid Ligand and a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound, a Lipid Construct comprising PDGF Nucleic Acid Ligand or a  
30 Complex comprising a PDGF Nucleic Acid Ligand and a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound.



PDGF is a prominent growth factor involved in restenosis. Restenosis, the reocclusion of a diseased blood vessel after treatment to eliminate stenosis, is a common occurrence that develops following coronary interventions and some peripheral vessel interventions.

Additionally, stents have been used in the treatment of or in conjunction with treatment of coronary and non-coronary vessels; however, restenosis is also associated with use of stents (called in-stent restenosis). In-stent restenosis occurs in about 15-30% of coronary interventions and frequently in some peripheral vessel interventions. For example, in-stent restenosis is a significant problem in small vessels, with frequencies ranging from 15% to 40% in stented femoral or popliteal arteries. Intermediate-sized vessels, such as renal arteries, have an in-stent restenosis rate of 10-20%.

The present invention, therefore, includes methods of treating, inhibiting, preventing or diagnosing restenosis by administration of a Complex comprising PDGF Nucleic Acid Ligand and a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound, a Lipid Construct comprising PDGF Nucleic Acid Ligand or a Complex comprising a PDGF Nucleic Acid Ligand and a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound. The present invention also includes methods of treating, inhibiting, preventing or diagnosing restenosis in coronary and non-coronary vessels. The present invention also includes methods of treating, inhibiting, preventing or diagnosing in-stent restenosis.

Additionally, cancer, angiogenesis, restenosis, and fibrosis involve the production of growth factors other than PDGF. Thus, it is contemplated by this invention that a Complex comprising PDGF Nucleic Acid Ligand and a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound, a Lipid Construct comprising PDGF Nucleic Acid Ligand or a Complex comprising a PDGF Nucleic Acid Ligand and a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound can be used in conjunction with Complexes comprising Nucleic Acid Ligands to other growth factors (such as bFGF, TGF $\beta$ , hKGF, etc.) and a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound, a Lipid Construct comprising PDGF Nucleic Acid Ligand or a Complex comprising a PDGF Nucleic Acid Ligand and a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound.

In one embodiment of the present invention, the Lipid Construct comprises a Complex comprised of a PDGF Nucleic Acid Ligand and a Lipophilic Compound with an additional

diagnostic or therapeutic agent encapsulated in the Lipid Construct or associated with the interior of the Lipid Construct. In the preferred embodiment, the Lipid Construct is a Lipid Bilayer Vesicle, and more preferably a Liposome. The therapeutic use of Liposomes includes the delivery of drugs which are normally toxic in the free form. In the liposomal form, the

5 toxic drug is occluded, and may be directed away from the tissues sensitive to the drug and targeted to selected areas. Liposomes can also be used therapeutically to release drugs over a prolonged period of time, reducing the frequency of administration. In addition, liposomes can provide a method for forming aqueous dispersions of hydrophobic or amphiphilic drugs, which are normally unsuitable for intravenous delivery.

10 In order for many drugs and imaging agents to have therapeutic or diagnostic potential, it is necessary for them to be delivered to the proper location in the body, and the liposome can thus be readily injected and form the basis for sustained release and drug delivery to specific cell types, or parts of the body. Several techniques can be employed to use liposomes to target encapsulated drugs to selected host tissues, and away from sensitive tissues. These techniques

15 include manipulating the size of the liposomes, their net surface charge, and their route of administration. MLVs, primarily because they are relatively large, are usually rapidly taken up by the reticuloendothelial system (principally the liver and spleen). UVs, on the other hand, have been found to exhibit increased circulation times, decreased clearance rates and greater biodistribution relative to MLVs.

20 Passive delivery of liposomes involves the use of various routes of administration, e.g., intravenous, subcutaneous, intramuscular and topical. Each route produces differences in localization of the liposomes. Two common methods used to direct liposomes actively to selected target areas involve attachment of either antibodies or specific receptor ligands to the surface of the liposomes. In one embodiment of the present invention, the PDGF Nucleic

25 Acid Ligand is associated with the outside surface of the liposome, and serves in a targeting capacity. Additional targeting components, such as antibodies or specific receptor ligands can be included on the liposome surface, as would be known to one of skill in the art. In addition, some efforts have been successful in targeting liposomes to tumors without the use of antibodies, *see*, for example, U.S. Patent No. 5,019,369, U.S. Patent No. 5,435,989, and U.S.

30 Patent No. 4,441,775, and it would be known to one of skill in the art to incorporate these alternative targeting methods.

Therapeutic or diagnostic compositions of a Complex comprising PDGF Nucleic Acid Ligand and a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound, a Lipid Construct comprising a Complex comprised of a PDGF Nucleic Acid Ligand and a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound, and a PDGF Nucleic Acid Ligand in association with a Lipid Construct without being part of a Complex may be administered parenterally by injection, although other effective administration forms, such as intraarticular injection, inhalant mists, orally active formulations, transdermal iontophoresis or suppositories, are also envisioned. They may also be applied locally by direct injection, can be released from devices, such as implanted stents or catheters, or delivered directly to the site by an infusion pump. One preferred carrier is physiological saline solution, but it is contemplated that other pharmaceutically acceptable carriers may also be used. In one embodiment, it is envisioned that the carrier and the PDGF Nucleic Acid Ligand Complex constitute a physiologically-compatible, slow release formulation. The primary solvent in such a carrier may be either aqueous or non-aqueous in nature. In addition, the carrier may contain other pharmacologically-acceptable excipients for modifying or maintaining the pH, osmolarity, viscosity, clarity, color, sterility, stability, rate of dissolution, or odor of the formulation. Similarly, the carrier may contain still other pharmacologically-acceptable excipients for modifying or maintaining the stability, rate of dissolution, release, or absorption of the PDGF Nucleic Acid Ligand. Such excipients are those substances usually and customarily employed to formulate dosages for parental administration in either unit dose or multi-dose form.

Once the therapeutic or diagnostic composition has been formulated, it may be stored in sterile vials as a solution, suspension, gel, emulsion, solid, or dehydrated or lyophilized powder. Such formulations may be stored either in ready to use form or requiring reconstitution immediately prior to administration. The manner of administering formulations containing PDGF Nucleic Acid Ligand for systemic delivery may be via subcutaneous, intramuscular, intravenous, intranasal or vaginal or rectal suppository.

The advantages of the Complexes and Lipid Constructs of the invention include: i) improving the plasma pharmacokinetics of the Nucleic Acid Ligand; ii) presenting Nucleic Acid Ligands in a multivalent array with the aim of increasing the avidity of interaction with their targets; iii) combining two or more presenting Nucleic Acid Ligands with different specificities in the same liposome particle; iv) enhancing the delivery of presenting Nucleic

Acid Ligands to tumors by taking advantage of the intrinsic tumor targeting properties of liposomes; and v) using the high affinity and specificity of presenting Nucleic Acid Ligands, which is comparable to that of antibodies, to guide liposomal contents to specific targets. Presenting Nucleic Acid Ligands are well suited for the kinds of preparations described here since, unlike most proteins, the denaturation of presenting Nucleic Acid Ligands by heat, various molecular denaturants and organic solvents is readily reversible.

The following examples are provided to explain and illustrate the present invention and are not to be taken as limiting of the invention. Example 1 describes the various materials and experimental procedures used in Examples 2-4 for the generation of ssDNA ligands to PDGF and tests associated therewith. Example 2 describes the ssDNA ligands to PDGF and the predicted secondary structure of selected nucleic acid ligands and a shared secondary structure motif. Example 3 describes the minimum sequence necessary for high affinity binding, the sites on the nucleic acid ligands and PDGF that are in contact, inhibition by DNA ligands of PDGF isoforms on cultured cells, and inhibition of mitogenic effects of PDGF in cells by DNA ligands. Example 4 describes substitutions of SELEX-derived ligands with modified nucleotides. Example 5 describes synthesis of PEG-modified PDGF Nucleic Acid Ligands. Example 6 describes stability of modified ligands in serum. Example 7 describes efficacy of a modified ligand (NX31975-40K PEG) in restenosis. Example 8 describes the various materials and method used in Example 9 for testing the inhibition of PDGF in glomerulonephritis. Example 9 describes inhibition of PDGF in glomerulonephritis. Example 10 describes the experimental procedures for evolving 2'-fluoro-2'-deoxypyrimidine RNA ligands to PDGF and the RNA sequences obtained.

#### EXAMPLE 1. EXPERIMENTAL PROCEDURES

This example provides the general procedures followed and incorporated in Examples 2-4.

##### MATERIALS.

Recombinant human PDGF-AA (Mr=29,000), PDGF-AB (Mr=27,000) and PDGF-BB (Mr=25,000) were purchased from R&D Systems (Minneapolis, MN) in lyophilized form, free from carrier protein. All three isoforms were produced in *E. coli* from synthetic genes based on the sequences for the long form of the mature human PDGF A-chain (Betsholtz *et al.* (1986) Nature 320:695-699) and the naturally occurring mature

form of human PDGF B-chain (Johnsson *et al.* (1984) EMBO J. 3:921-928). Randomized DNA libraries, PCR primers and DNA ligands and 5'-iodo-2'-deoxyuridine-substituted DNA ligands were synthesized by NeXstar Pharmaceuticals, Inc. (Boulder, CO) or by Operon Technologies (Alameda, CA) using the standard solid phase phosphoramidite method (Sinha *et al.* (1984) Nucleic Acids Res. 12:4539-4557).

#### SINGLE STRANDED DNA (ssDNA) SELEX

Essential features of the SELEX procedure have been described in detail in the SELEX Patent Applications (see also Tuerk and Gold (1990) Science 249:505; Jellinek *et al.* (1994) Biochemistry 33:10450; Jellinek *et al.* (1993) Proc. Natl. Acad. Sci. USA 90:11227), which are incorporated by reference herein. The initial ssDNA library containing a contiguous randomized region of forty nucleotides, flanked by primer annealing regions (Table 1) (SEQ ID NOS:1-3) of invariant sequence, was synthesized by the solid phase phosphoramidite method using equal molar mixture of the four phosphoramidites to generate the randomized positions. The ssDNA library was purified by electrophoresis on an 8% polyacrylamide/7 M urea gel. The band that corresponds to the full-length DNA was visualized under UV light, excised from the gel, eluted by the crush and soak method, ethanol precipitated and pelleted by centrifugation. The pellet was dried under vacuum and resuspended in phosphate buffered saline supplemented with 1 mM MgCl<sub>2</sub> (PBSM = 10.1 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, 137 mM NaCl and 2.7 mM KCl, 1 mM MgCl<sub>2</sub>, pH 7.4) buffer. Prior to incubation with the protein, the ssDNA was heated at 90°C for 2 minutes in PBSM and cooled on ice. The first selection was initiated by incubating approximately 500 pmol (3 x 10<sup>14</sup> molecules) of 5' <sup>32</sup>P end-labeled random ssDNA with PDGF-AB in binding buffer (PBSM containing 0.01% human serum albumin (HSA)). The mixture was incubated at 4°C overnight, followed by a brief (15 min) incubation at 37°C. The DNA bound to PDGF-AB was separated from unbound DNA by electrophoresis on an 8% polyacrylamide gel (1:30 bis-acrylamide:acrylamide) at 4°C and at 5 V/cm with 89 mM Tris-borate (pH 8.3) containing 2 mM EDTA as the running buffer. The band that corresponds to the PDGF-ssDNA complex, which runs with about half the electrophoretic mobility of the free ssDNA, was visualized by autoradiography, excised from the gel and eluted by the crush and soak method. In subsequent affinity selections, the ssDNA was incubated with PDGF-AB for 15 minutes at 37°C in binding buffer and the

PDGF-bound ssDNA was separated from the unbound DNA by nitrocellulose filtration, as previously described (Green *et al.* (1995) Chemistry and Biology 2:683-695). All affinity-selected ssDNA pools were amplified by PCR in which the DNA was subjected to 12-20 rounds of thermal cycling (30 s at 93°C, 10 s at 52°C, 60 s at 72°C) in 10 mM Tris-Cl (pH 8.4) containing 50 mM KCl, 7.5 mM MgCl<sub>2</sub>, 0.05 mg/ml bovine serum albumin, 1 mM deoxynucleoside triphosphates, 5 μM primers (Table 1) (SEQ ID NOS:2, 3) and 0.1 units/μl Taq polymerase. The 5' PCR primer was 5' end-labeled with polynucleotide kinase and [α-<sup>32</sup>P]ATP and the 3' PCR primer was biotinylated at the 5' end using biotin phosphoramidite (Glen Research, Sterling, VA). Following PCR amplification, streptavidin (Pierce, Rockford, IL) was added to the unpurified PCR reaction mixture at a 10-fold molar excess over the biotinylated primer and incubated for 15 min at room temperature. The dsDNA was denatured by adding an equal volume of stop solution (90% formamide, 1% sodium dodecyl sulfate, 0.025% bromophenol blue and xylene cyanol) and incubating for 20 min at room temperature. The radiolabeled strand was separated from the streptavidin-bound biotinylated strand by electrophoresis on 12% polyacrylamide/7 M urea gels. The faster migrating radiolabeled (non-biotinylated) ssDNA strand was cut out of the gel and recovered as described above. The amount of ssDNA was estimated from the absorbance at 260 nm using the extinction coefficient of 33 μg/ml/absorbance unit (Sambrook *et al.* (1989) in Molecular Cloning: A Laboratory Manual, 2nd Ed. 3 vols., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York).

Cloning and Sequencing. The amplified affinity-enriched pool from SELEX round 12 was purified on a 12% polyacrylamide gel and cloned between *Hind*III and *Pst*I sites in JM109 strain of *E. coli* (Sambrook *et al.* (1989) in Molecular Cloning: A Laboratory Manual, 2nd Ed. 3 vols., Cold Spring Harbor Laboratory Press, Cold Spring Harbor). Individual clones were used to prepare plasmids by alkaline lysis. Plasmids were sequenced at the insert region using the forward sequencing primer and Sequenase 2.0 (Amersham, Arlington Heights, IL) according to the manufacturer's protocol.

Determination of the apparent equilibrium dissociation constants and the dissociation rate constants. The binding of ssDNA ligands at low concentrations to varying concentrations of PDGF was determined by the nitrocellulose filter binding method as described (Green *et al.*

(1995) Chemistry and Biology 2:683-695). The concentrations of PDGF stock solutions (in PBS) were determined from the absorbance readings at 280 nm using the following  $\epsilon_{280}$  values calculated from the amino acid sequences (Gill and von Hippel (1989) Anal. Biochem. 182:319-326): 19,500 M<sup>-1</sup>cm<sup>-1</sup> for PDGF-AA, 15,700 M<sup>-1</sup>cm<sup>-1</sup> for PDGF-AB and 11,800 M<sup>-1</sup>cm<sup>-1</sup> for PDGF-BB. ssDNA for all binding experiments were purified by electrophoresis on 8% (>80 nucleotides) or 12% (<40 nucleotides) polyacrylamide/7 M urea gels. All ssDNA ligands were heated at 90°C in binding buffer at high dilution ( $\approx 1$  nM) for 2 min and cooled on ice prior to further dilution into the protein solution. The binding mixtures were typically incubated for 15 min at 37°C before partitioning on nitrocellulose filters:

The binding of DNA ligands (L) to PDGF-AA (P) is adequately described with the bimolecular binding model for which the fraction of bound DNA at equilibrium (q) is given by equation 1:

$$q = (f/2[L]_t) \{ [P]_t + [L]_t + K_d - [(P]_t + [L]_t + K_d)^2 - 4[P]_t[L]_t \}^{1/2} \quad (1)$$

where  $[P]_t$  and  $[R]_t$  are total protein and total DNA concentrations,  $K_d$  is the equilibrium dissociation constant and  $f$  is the efficiency of retention of protein-DNA complexes on nitrocellulose filters (Irvine *et al.* (1991) J. Mol. Biol. 222:739-761; Jellinek *et al.* (1993) Proc. Natl. Acad. Sci. USA 90:11227-11231).

The binding of DNA ligands to PDGF-AB and PDGF-BB is biphasic and can be described by a model in which the DNA ligand is composed of two non-interconverting components ( $L_1$  and  $L_2$ ) that bind to the protein with different affinities, described by corresponding dissociation constants,  $K_{d1}$  and  $K_{d2}$  (Jellinek *et al.* (1993) Proc. Natl. Acad. Sci. USA 90:11227-11231). In this case, the explicit solution for the fraction of bound DNA (q) is given by equation 2:

$$q = f \left( \frac{\chi_1 K_{d1}}{1 + K_{d1} [P]} + \frac{\chi_2 K_{d2}}{1 + K_{d2} [P]} \right) [P] \quad (2)$$

30 with

$$P] = \frac{[P] t}{1 + \frac{\chi_1 K_{d1} [L] t}{1 + K_{d1} [P]} + \frac{\chi_2 K_{d2} [L] t}{1 + K_{d2} [P]}}$$

where  $\chi_1$  and  $\chi_2 (=1-\chi_1)$  are the mole fractions of  $L_1$  and  $L_2$ . The  $K_d$  values for the binding of DNA ligands to PDGF were calculated by fitting the data points to equation 1 (for

- 5 PDGF-AA) or equation 2 (for PDGF-AB and PDGF-BB) using the non-linear least squares method.

The dissociation rate constants ( $k_{off}$ ) were determined by measuring the amount of  $^{32}P$  5'-end labeled minimal ligands (0.17 nM) bound to PDGF-AB (1 nM) as a function of time following the addition of 500-fold excess of unlabeled ligands, using nitrocellulose  
10 filter binding as the partitioning method. The  $k_{off}$  values were determined by fitting the data points to the first-order rate equation 3:

$$(q-q_{\infty})/(q_0-q_{\infty}) = \exp(-k_{off}t) \quad (3)$$

15

where  $q$ ,  $q_0$  and  $q_{\infty}$  represent the fractions of DNA bound to PDGF-AB at any time ( $t$ ),  $t=0$  and  $t=\infty$ , respectively.

Minimal ligand determinations. To generate a population of 5' end-labeled DNA ligands  
20 serially truncated from the 3' end, a primer complementary to the 3' invariant sequence region of a DNA ligand template (truncated primer 5N2, Table 1) (SEQ ID NO:3) was radiolabeled at the 5' end with  $[\gamma-^{32}P]$ -ATP and T4 polynucleotide kinase, annealed to the template and extended with Sequenase (Amersham, Arlington Heights, IL) and a mixture of all four dNTPs and ddNTPs. Following incubation in binding buffer for 15 min at 37°C, the  
25 fragments from this population that retain high affinity binding to PDGF-AB were separated from those with weaker affinity by nitrocellulose filter partitioning. Electrophoretic resolution of the fragments on 8% polyacrylamide/7 M urea gels, before and after affinity selection, allows determination of the 3' boundary. To generate a population of 3' end-labeled DNA ligands serially truncated from the 5' end, the DNA ligands were  
30 radiolabeled at the 3' end with  $[\alpha-^{32}P]$ -cordycepin-5'-triphosphate (New England Nuclear,



Boston, MA) and T4 RNA ligase (Promega, Madison, WI), phosphorylated at the 5' end with ATP and T4 polynucleotide kinase, and partially digested with lambda exonuclease (Gibco BRL, Gaithersburg, MD). Partial digestion of 10 pmols of 3'-labeled ligand was done in 100  $\mu$ L volume with 7 mM glycine-KOH (pH 9.4), 2.5 mM  $MgCl_2$ , 1  $\mu$ g/ml BSA, 5 15  $\mu$ g tRNA, and 4 units of lambda exonuclease for 15 min at 37°C. The 5' boundary was determined in an analogous manner to that described for the 3' boundary.

Melting temperature ( $T_m$ ) measurements. Melting profiles for the minimal DNA ligands were obtained on a Cary Model 1E spectrophotometer. Oligonucleotides (320-400 nM) 10 were heated to 95°C in PBS, PBSM or PBS with 1 mM EDTA and cooled to room temperature prior to the melting profile determination. Melting profiles were generated by heating the samples at the rate of 1°C/min from 15-95°C and recording the absorbance every 0.1°C. The first derivative of the data points was calculated using the plotting program KaleidaGraph (Synergy Software, Reading, PA). The first derivative values were 15 smoothed using a 55 point smoothing function by averaging each point with 27 data points on each side. The peak of the smoothed first derivative curves was used to estimate the  $T_m$  values.

Crosslinking of 5-iodo-2'-deoxyuridine-substituted DNA ligands to PDGF-AB. DNA 20 ligands containing single or multiple substitutions of 5'-iodo-2'-deoxyuridine for thymidine were synthesized using the solid phase phosphoramidite method. To test for the ability to crosslink, trace amounts of 5'  $^{32}P$  end-labeled ligands were incubated with PDGF-AB (100 nM) in binding buffer at 37°C for 15 min prior to irradiation. The binding mixture was transferred to a 1 cm path length cuvette thermostated at 37°C and irradiated at 308 nm for 25 25-400 s at 20 Hz using a XeCl charged Lumonics Model EX748 excimer laser. The cuvette was positioned 24 cm beyond the focal point of a convergent lens, with the energy at the focal point measuring 175 mJoules/pulse. Following irradiation, aliquots were mixed with an equal volume of formamide loading buffer containing 0.1% SDS and incubated at 95°C for 5 min prior to resolution of the crosslinked PDGF/ligand complex from the free 30 ligand on 8% polyacrylamide/7 M urea gels.

To identify the protein site of crosslinking for ligand 20t-I4 (SEQ ID NO:92), binding and irradiation were done on a larger scale. PDGF-AB and 5'  $^{32}P$  end-labeled ligand, each at 1  $\mu$ M in PBSM, were incubated and irradiated (300 s) as described above in

two 1 ml reaction vessels. The reaction mixtures were combined, ethanol precipitated and resuspended in 0.3 ml of Tris-HCl buffer (100 mM, pH 8.5). The PDGF-AB/ligand crosslinked complex was digested with 0.17  $\mu\text{g}/\mu\text{l}$  of modified trypsin (Boehringer Mannheim) for 20 hours at 37°C. The digest mixture was extracted with  
5 phenol/chloroform, chloroform and then ethanol precipitated. The pellet was resuspended in water and an equal volume of formamide loading buffer with 5% (v/v)  $\beta$ -mercaptoethanol (no SDS), incubated at 95°C for 5 min, and resolved on a 40 cm 8% polyacrylamide/7 M urea gel. The crosslinked tryptic-peptide/ligand that migrated as two closely spaced bands about 1.5 cm above the free ligand band was excised from the gel and eluted by the crush  
10 and soak method and ethanol precipitated. The dried crosslinked peptide (about 160 pmoles based on the specific activity) was sequenced by Edman degradation (Midwest Analytical, Inc., St. Louis, MO).

Receptor Binding Assay. The binding of  $^{125}\text{I}$ -PDGF-AA and  $^{125}\text{I}$ -PDGF-BB to porcine  
15 aortic endothelial (PAE) cells transfected with PDGF  $\alpha$ - or  $\beta$ -receptors were performed as described (Heldin *et al.*, (1988) EMBO J. 7:1387-1394). Different concentrations of DNA ligands were added to the cell culture (1.5  $\text{cm}^2$ ) in 0.2 ml of phosphate buffered saline supplemented with 1 mg bovine serum albumin per ml together with  $^{125}\text{I}$ -PDGF-AA (2 ng, 100,000 cpm) or  $^{125}\text{I}$ -PDGF-BB (2 ng, 100,000 cpm). After incubation at 4°C for 90  
20 minutes, the cell cultures were washed and cell associated radioactivity determined in a g-counter (Heldin *et al.*, (1988) EMBO J. 7:1387-1394).

[ $^3\text{H}$ ]thymidine Incorporation Assay. The incorporation of [ $^3\text{H}$ ]thymidine into PAE cells expressing PDGF  $\beta$ -receptor in response to 20 ng/ml of PDGF-BB or 10% fetal calf serum  
25 and in the presence of different concentrations of DNA ligands was performed as described (Mori *et al.* (1991) J. Biol. Chem. 266:21158-21164). After incubation for 24 hours at 37°C,  $^3\text{H}$ -radioactivity incorporated into DNA was determined using a  $\beta$ -counter.

## EXAMPLE 2. ssDNA LIGANDS OF PDGF

30 High affinity DNA ligands to PDGF-AB were identified by the SELEX process from a library of  $\approx 3 \times 10^{14}$  molecules (500 pmol) of single stranded DNA randomized at forty contiguous positions (Table 1) (SEQ ID NO:1). The PDGF-bound DNA was

separated from unbound DNA by polyacrylamide gel electrophoresis in the first round and by nitrocellulose filter binding in the subsequent rounds. After 12 rounds of SELEX, the affinity-enriched pool bound to PDGF-AB with an apparent dissociation constant ( $K_d$ ) of  $\approx 50$  pM (data not shown). This represented an improvement in affinity of  $\approx 700$ -fold compared to the initial randomized DNA library. This affinity-enriched pool was used to generate a cloning library from which 39 isolates were sequenced. Thirty-two of these ligands were found to have unique sequences (Table 2) (SEQ ID NOS:4-35). Ligands that were subjected to the minimal sequence determination are marked with an asterisk (\*) next to the clone number. The clone numbers that were found to retain high affinity binding as minimal ligands are italicized. All ligands shown in Table 2 were screened for their ability to bind to PDGF-AB using the nitrocellulose filter binding method. To identify the best ligands from this group, the relative affinities for PDGF-AB were determined by measuring the fraction of 5'  $^{32}$ P end-labeled ligands bound to PDGF-AB over a range of protein concentrations. For the ligands that bound to PDGF-AB with high affinity, the affinity toward PDGF-BB and PDGF-AA was also examined. In all cases, the affinity of ligands for PDGF-AB and PDGF-BB was comparable while the affinity for PDGF-AA was considerably lower (data not shown).

Twenty-one of the thirty-two unique ligands can be grouped into a sequence family shown in Table 3 (SEQ ID NOS:4, 5, 7-9, 14-24, 26, 31, 32, 34 and 35). The sequences of the initially randomized region (uppercase letters) are aligned according to the consensus three-way helix junction motif. Nucleotides in the sequence-invariant region (lowercase letters) are only shown where they participate in the predicted secondary structure. Several ligands were "disconnected" (equality symbol) in order to show their relatedness to the consensus motif through circular permutation. The nucleotides predicted to participate in base pairing are indicated with underline inverted arrows, with the arrow heads pointing toward the helix junction. The sequences are divided into two groups, A and B, based on the first single stranded nucleotide (from the 5' end) at the helix junction (A or G, between helices II and III). Mismatches in the helical regions are shown with dots under the corresponding letters (G-T and T-G base pairs were allowed). In places where single nucleotide bulges occur, the mismatched nucleotide is shown above the rest of the sequence between its neighbors.

This classification is based in part on sequence homology among these ligands, but

in greater part on the basis of a shared secondary structure motif: a three-way helix junction with a three nucleotide loop at the branch point (Figure 1) (SEQ ID NO:82). These ligands were subdivided into two groups; for ligands in group A, the loop at the branch point has an invariant sequence AGC and in group B, that sequence is G(T/G)(C/T). The proposed  
5 consensus secondary structure motif is supported by base-pairing covariation at non-conserved nucleotides in the helices (Table 4). Since the three-way junctions are encoded in continuous DNA strands, two of the helices end in loops at the distal end from the junction. These loops are highly variable, both in length and in sequence. Furthermore, through circular permutation of the consensus motif, the loops occur in all three helices,  
10 although they are most frequent in helices II and III. Together these observations suggest that the regions distal from the helix junction are not important for high affinity binding to PDGF-AB. The highly conserved nucleotides are indeed found near the helix junction (Table 3, Figure 1).

### 15 **EXAMPLE 3. MINIMAL LIGAND DETERMINATIONS**

The minimal sequence necessary for high affinity binding was determined for six of the best ligands to PDGF-AB. In general, the information about the 3' and 5' minimal sequence boundaries can be obtained by partially fragmenting the nucleic acid ligand and then selecting for the fragments that retain high affinity for the target. With RNA ligands,  
20 the fragments can be conveniently generated by mild alkaline hydrolysis (Tuerk *et al.* (1990) *J. Mol. Biol.* 213:749-761; Jellinek *et al.* (1994) *Biochemistry* 33:10450-10456; Jellinek *et al.* (1995) *Biochemistry* 34:11363-11372; Green *et al.* (1995) *J. Mol. Biol.* 247:60-68). Since DNA is more resistant to base, an alternative method of generating fragments is needed for DNA. To determine the 3' boundary, a population of ligand  
25 fragments serially truncated at the 3' end was generated by extending the 5' end-labeled primer annealed to the 3' invariant sequence of a DNA ligand using the dideoxy sequencing method. This population was affinity-selected by nitrocellulose filtration and the shortest fragments (truncated from the 3' end) that retain high affinity binding for PDGF-AB were identified by polyacrylamide gel electrophoresis. The 5' boundary was determined in an  
30 analogous manner except that a population of 3' end-labeled ligand fragments serially truncated at the 5' end was generated by limited digestion with lambda exonuclease. The minimal ligand is then defined as the sequence between the two boundaries. It is important

to keep in mind that, while the information derived from these experiments is useful, the suggested boundaries are by no means absolute since the boundaries are examined one terminus at the time. The untruncated (radiolabeled) termini can augment, reduce or have no effect on binding (Jellinek *et al.* (1994) *Biochemistry* 33:10450-10456).

5        Of the six minimal ligands for which the boundaries were determined experimentally, two (20t (SEQ ID NO:83) (Figure 2A) and 41t (SEQ ID NO:85) (Figure 2C); truncated versions of ligands 20 and 41) bound with affinities comparable (within a factor of 2) to their full-length analogs and four had considerably lower affinities. The two minimal ligands that retained high affinity binding to PDGF, 20t and 41t, contain the  
10       predicted three-way helix junction secondary structure motif (Figures 2A-C) (SEQ ID NOS:83-85). The sequence of the third minimal ligand that binds to PDGF-AB with high affinity, 36t (SEQ ID NO:84), was deduced from the knowledge of the consensus motif (Figure 2B). In subsequent experiments, we found that the single-stranded region at the 5' end of ligand 20t is not important for high affinity binding. Furthermore, the trinucleotide  
15       loops on helices II and III in ligand 36t (GCA and CCA) can be replaced with hexaethylene glycol spacers (*infra*). These experiments provide further support for the importance of the helix junction region in high affinity binding to PDGF-AB.

Binding of the Minimal Ligands to PDGF. The binding of minimal ligands 20t, 36t, and 41t  
20       to varying concentrations of PDGF-AA, PDGF-AB and PDGF-BB is shown in Figures 3A-3C. In agreement with the binding properties of their full length analogs, the minimal ligands bind to PDGF-AB and PDGF-BB with substantially higher affinity than to PDGF-AA (Figures 3A-3C, Table 4). In fact, their affinity for PDGF-AA is comparable to that of random DNA (data not shown). The binding to PDGF-AA is adequately described with a  
25       monophasic binding equation while the binding to PDGF-AB and PDGF-BB is notably biphasic. In previous SELEX experiments, biphasic binding has been found to be a consequence of the existence of separable nucleic acid species that bind to their target protein with different affinities (Jellinek *et al.* (1995) *Biochemistry* 34:11363-11372) and unpublished results). The identity of the high and the low affinity fractions is at present not  
30       known. Since these DNA ligands described here were synthesized chemically, it is possible that the fraction that binds to PDGF-AB and PDGF-BB with lower affinity represents chemically imperfect DNA. Alternatively, the high and the low affinity species may

represent stable conformational isomers that bind to the PDGF B-chain with different affinities. In any event, the higher affinity binding component is the most populated ligand species in all cases (Figures 3A-3C). For comparison, a 39-mer DNA ligand that binds to human thrombin with a  $K_d$  of 0.5 nM (ligand T39 (SEQ ID NO:88)):

- 5 5'-CAGTCCGTGGTAGGGCAGGTTGGGGTGA CTTCGTGGAA[3'T], where [3'T] represents a 3'-3' linked thymidine nucleotide added to reduce 3'-exonuclease degradation) and has a predicted stem-loop structure, binds to PDGF-AB with a  $K_d$  of 0.23  $\mu$ M (data not shown).

- 10 Dissociation Rates of the Minimal Ligands. To evaluate the kinetic stability of the PDGF-AB/DNA complexes, the dissociation rates at 37°C for the complexes of minimal ligands 20t (SEQ ID NO:83), 36t (SEQ ID NO:84) and 41t (SEQ ID NO:85) with PDGF-AB were determined by measuring the amount of radiolabeled ligands (0.17 nM) bound to PDGF-AB (1 nM) as a function of time following the addition of a large excess of
- 15 unlabeled ligands (Figure 4). At these protein and DNA ligand concentrations, only the high affinity fraction of the DNA ligands binds to PDGF-AB. The following values for the dissociation rate constants were obtained by fitting the data points shown in Figure 4 to the first-order rate equation:  $4.5 \pm 0.2 \times 10^{-3} \text{ s}^{-1}$  ( $t_{1/2} = 2.6 \text{ min}$ ) for ligand 20t,  $3.0 \pm 0.2 \times 10^{-3} \text{ s}^{-1}$  ( $t_{1/2} = 3.8 \text{ min}$ ) for ligand 36t, and  $1.7 \pm 0.1 \times 10^{-3} \text{ s}^{-1}$  ( $t_{1/2} = 6.7 \text{ min}$ ) for ligand 41t. The
- 20 association rates calculated for the dissociation constants and dissociation rate constants ( $k_{on}=k_{off}/K_d$ ) are  $3.1 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$  for 20t,  $3.1 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$  for 36t and  $1.2 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$  for 41t.

- Melting Temperatures of the Minimal Ligands. Melting temperatures ( $T_m$ 's) were determined for minimal ligands 20t, 36t and 41t from the UV absorption vs. temperature
- 25 profiles (Figure 5). At the oligonucleotide concentrations used in these experiments (320-440 nM), only the monomeric species were observed as single bands on non-denaturing polyacrylamide gels. The  $T_m$  values were obtained from the first derivative replots of the melting profiles. Ligands 20t and 41t exhibited monophasic melting with  $T_m$  values of 44°C and 49°C. The melting profile of ligand 36t was biphasic, with the  $T_m$  value
- 30 of 44°C for the first (major) transition and  $\approx 63^\circ\text{C}$  for the second transition.

Photocrosslinking of 5-Iodo-2'-Deoxyuridine Substituted Minimal DNA Ligands to PDGF-

AB. To determine the sites on the DNA ligands and PDGF that are in close contact, a series of photo-crosslinking experiments was performed with 5'-iodo-2'-deoxyuridine

(IdU)-substituted DNA ligands 20t, 36t and 41t. Upon monochromatic excitation at 308

5 nm, 5-iodo- and 5-bromo-substituted pyrimidine nucleotides populate a reactive triplet state following intersystem crossing from the initial  $n$  to  $\pi^*$  transition. The excited triplet state species then reacts with electron rich amino acid residues (such as Trp, Tyr and His) that are in its close proximity to yield a covalent crosslink. This method has been used extensively in studies of nucleic acid-protein interactions since it allows irradiation with >300 nm light  
10 which minimizes photodamage (Willis *et al.* (1994) *Nucleic Acids Res.* 22:4947-4952; Stump and Hall (1995) *RNA* 1:55-63; Willis *et al.* (1993) *Science* 262:1255-1257; Jensen *et al.* (1995) *Proc. Natl. Acad. Sci. USA* 92:12220-12224). Analogs of ligands 20t, 36t and 41t were synthesized in which all thymidine residues were replaced with IdU residues using the solid phase phosphoramidite method. The affinity of these IdU-substituted ligands for  
15 PDGF-AB was somewhat enhanced compared to the unsubstituted ligands and based on the appearance of bands with slower electrophoretic mobility on 8% polyacrylamide/7 M urea gels, all three 5' end-labeled IdU-substituted ligands crosslinked to PDGF-AB upon irradiation at 308 nm (data not shown). The highest crosslinking efficiency was observed with IdU-substituted ligand 20t. To identify the specific IdU position(s) responsible for the  
20 observed crosslinking, seven singly or multiply IdU-substituted analogs of 20t were tested for their ability to photo-crosslink to PDGF-AB: ligands 20t-I1 through 20t-I7 (5'-TGGGAGGGCGCGT<sup>1</sup>T<sup>1</sup>CT<sup>1</sup>T<sup>1</sup>CGT<sup>2</sup>GGT<sup>3</sup>T<sup>4</sup>ACT<sup>5</sup>T<sup>6</sup>T<sup>6</sup>AGT<sup>7</sup>CCCG-3' (SEQ ID NOS:89-95) where the numbers indicate IdU substitutions at indicated thymidine nucleotides for the seven ligands). Of these seven ligands, efficient crosslinking to  
25 PDGF-AB was observed only with ligand 20t-I4 (SEQ ID NO:92). The photo-reactive IdU position corresponds to the 3' proximal thymidine in the loop at the helix junction (Figure 2).

To identify the crosslinked amino acid residue(s) on PDGF-AB, a mixture of 5' end-labeled 20t-I4 and PDGF-AB was incubated for 15 min at 37°C followed by irradiation  
30 at 308 nm. The reaction mixture was then digested with modified trypsin and the crosslinked fragments resolved on an 8% polyacrylamide/7 M urea gel. Edman degradation of the peptide fragment recovered from the band that migrated closest to the free DNA band

revealed the amino acid sequence KKPIXKK (SEQ ID NO:96), where X indicates a modified amino acid that could not be identified with the 20 derivatized amino acid standards. This peptide sequence, where X is phenylalanine, corresponds to amino acids 80-86 in the PDGF-B chain (Johnsson *et al.* (1984) EMBO J. 3:921-928) which in the crystal structure of PDGF-BB comprises a part of solvent-exposed loop III (Oefner *et al.* (1992) EMBO J. 11:3921-3926). In the PDGF A-chain, this peptide sequence does not occur (Betsholtz *et al.* (1986) Nature 320:695-699). Together, these data establish a point contact between a specific thymidine residue in ligand 20t and phenylalanine 84 of the PDGF B-chain.

Receptor Binding Assay. In order to determine whether the DNA ligands to PDGF were able to inhibit the effects of PDGF isoforms on cultured cells, the effects on binding of <sup>125</sup>I-labeled PDGF isoforms to PDGF  $\alpha$ - and  $\beta$ -receptors stably expressed in porcine aortic endothelial (PAE) cells by transfection were first determined. Ligands 20t, 36t and 41t all efficiently inhibited the binding of <sup>125</sup>I-PDGF-BB to PDGF  $\alpha$ -receptors (Figure 6) or PDGF  $\beta$ -receptors (data not shown), with half maximal effects around 1 nM of DNA ligand. DNA ligand T39 (SEQ ID NO:88) (described *supra*), directed against thrombin and included as a control, showed no effect. None of the ligands was able to inhibit the binding of <sup>125</sup>I-PDGF-AA to the PDGF  $\alpha$ -receptor (data not shown), consistent with the observed specificity of ligands 20t, 36t and 41t for PDGF-BB and PDGF-AB.

Inhibition of Mitogenic Effects by Minimal Ligands. The ability of the DNA ligands to inhibit the mitogenic effects of PDGF-BB on PAE cells expressing PDGF  $\beta$ -receptors was investigated. As shown in Figure 7, the stimulatory effect of PDGF-BB on [<sup>3</sup>H]thymidine incorporation was neutralized by ligands 20t, 36t and 41t. Ligand 36t exhibited half maximal inhibition at the concentration of 2.5 nM; ligands 41t was slightly more efficient and 20t slightly less efficient. The control ligand T39 had no effect. Moreover, none of the ligands inhibited the stimulatory effects of fetal calf serum on [<sup>3</sup>H]thymidine incorporation in these cells, showing that the inhibitory effects are specific for PDGF.

#### EXAMPLE 4. POST-SELEX MODIFICATIONS

The stability of nucleic acids to nucleases is an important consideration in efforts to develop nucleic acid-based therapeutics. Experiments have shown that many, and in some



cases most of the nucleotides in SELEX-derived ligands can be substituted with modified nucleotides that resist nuclease digestion, without compromising high affinity binding (Green *et al.* (1995) Chemistry and Biology 2:683-695; Green *et al.* (1995) J. Mol. Biol. 247:60-68).

5 A series of substitution experiments were conducted to identify positions in ligand 36t that tolerate 2'-O-methyl (2'-O-Me) or 2'-fluoro (2'-F) substitution. Tables 6 and 7 and Figures 8A and 8B summarize the substitutions examined and their effect on the affinity of the modified ligands for PDGF-AB or PDGF-BB. 2-Fluoropyrimidine nucleoside phosphoramidites were obtained from JBL Scientific (San Louis Obispo, CA). 2'-O-  
10 Methylpurine phosphoramidites were obtained from PerSeptive Biosystems (Boston, MA). All other nucleoside phosphoramidites were from PerSeptive Biosystems (Boston, MA). Not all substitution combinations were examined. Nevertheless, these experiments have been used to identify the pattern of 2'-O-Me and 2'-F substitutions that are compatible with high affinity binding to PDGF-AB or PDGF-BB. It is worth noting that trinucleotide loops  
15 on helices II and III in ligand 36t (Figures 2B and 8B) can be replaced with pentaethylene glycol (18-atom) spacers (Spacer Phosphoramidite 18, Glen Research, Sterling, VA) (see Example 5 for description of synthesis of pentaethylene glycol-substituted ligand) without compromising high affinity binding to PDGF-AB or -BB. This is in agreement with the notion that the helix junction domain of the ligand represents the core of the structural motif  
20 required for high affinity binding. In practical terms, the replacement of six nucleotides with two pentaethylene glycol spacers is advantageous in that it reduces by four the number of coupling steps required for the synthesis of the ligand. In addition to the substitution experiments, four nucleotides from the base of helix I were found that could be deleted without loss of binding affinity (compare for example ligand 36t (SEQ ID NO:84) with 36ta  
25 (SEQ ID NO:141) or ligand 1266 (SEQ ID NO:124) with 1295 (SEQ ID NO:127) in Tables 6 and 7).

## EXAMPLE 5. SYNTHESIS OF PEG-MODIFIED PDGF NUCLEIC ACID LIGANDS

### A) General Procedure for the Synthesis of NX31975 (SEQ ID NO:148) on Solid Support

5        Synthesis was carried out on a 1 mmol scale on a millipore 8800 automated synthesizer using standard deoxynucleoside phosphoramidites, 2'-O-methyl-5'-O-DMT-N2-tert-butylphenoxyacetylguanosine-phosphoramidite, 2'-O-methyl-5'-O-DMT-N6-tert-butylphenoxyacetyl-adenosine-phosphoramidite, 2'-deoxy-2'-fluoro-5'-O-DMT-uridine-phosphoramidite, 2'-deoxy-2'-fluoro-5'-O-DMT-N4-acetylcytidine-3'-N,N-diisopropyl-(2-  
10 cyanoethyl)-phosphoramidite, 18-O-DMT-hexaethyleneglycol-1-[N,N-diisopropyl-(2-cyanoethyl)-phosphoramidite] (Figure 9C), and 5-trifloroacetamidopentane-1-[N,N-diisopropyl-(2-cyanoethyl)-phosphoramidite] (Figure 9D). The syntheses were carried out using 4,5-dicyanoimidazole as the activator on controlled pore glass (CPG) support of 600A pore size, 80-120 mesh, and 60 -70  $\mu\text{mol/g}$  loading with 5'-succinyl thymidine. After the  
15 synthesis, the oligos were deprotected with 40%  $\text{NH}_4\text{OH}$ , at 55°C for 16 hours. The support was filtered, and washed with water and 1:1 acetonitrile/water and the combined washings were evaporated to dryness. The ammonium counterion on the backbone was exchanged for triethylammonium ion by reverse phase salt exchange and the solvent was evaporated to afford the crude oligo as the triethylammonium salt.

20        Hexaethylene glycol spacers on the loops are attached to the nucleotides through phosphate linkages. The structures of the 2 loops are shown in Figures 9A and 9B. The 5' phosphate group shown is from the hexaethylene glycol phosphoramidite.

### B) Conjugation of 40K PEG NHS ester to the aminolinker on PDGF Nucleic Acid Ligands

25        The NX31975 crude oligonucleotide containing the 5' primary amino group was dissolved in 100 mM sodium borate buffer (pH 9) to 60 mg /ml concentration. In a separate tube 2 equivalents of PEG NHS ester (Figure 9E) (Shearwater Polymers, Inc.) was dissolved in dry DMF (Ratio of borate : DMF 1:1) and the mixture was warmed to dissolve the PEG NHS ester. Then the oligo solution was quickly added to PEG solution and the mixture was  
30 vigorously stirred at room temperature for 10 minutes. About 95% of the oligo conjugated to the PEG NHS ester.

**EXAMPLE 6. STABILITY OF MODIFIED LIGANDS IN SERUM**

The stabilities of DNA (36ta) (SEQ ID NO:141) and modified DNA (NX21568) (SEQ ID NO:146) ligands in rat serum at 37°C were compared. Serum used for these experiments was obtained from a Sprague-Dawley rat and was filtered through 0.45 µm cellulose acetate filter and buffered with 20 mM sodium phosphate buffer. Test ligands (36ta or NX21568) were added to the serum at the final concentration of 500 nM. The final serum concentration was 85% as a result of the addition of buffer and ligand. From the original 900 µl incubation mixture, 100 µl aliquots were withdrawn at various time points and added to 10 µl of 500 mM EDTA (pH 8.0), vortexed and frozen on dry ice and stored at -20°C until the end of the experiment. The amount of full length oligonucleotide ligand remaining for each of the time points was quantitated by HPLC analysis. To prepare the samples for HPLC injections, 200 µl of a mixture of 30% formamide, 70% 25 mM Tris buffer (pH 8.0) containing 1% acetonitrile was added to 100 µl of thawed time point samples, vortexed for 5 seconds and centrifuged for 20 minutes at 14,000 rpm in an Eppendorf microcentrifuge. The analysis was performed using an anion exchange chromatography column (NuceoPac, Dionex, PA-100, 4x50 mm) applying a LiCl gradient. The amount of full length oligonucleotide remaining at each time point was determined from the peak areas (Figure 10). With a half-life of about 500 minutes, the modified ligand (NX21568) exhibited a substantially greater stability in rat serum compared with the DNA ligand (36ta), which was degraded with a half-life of about 35 minutes (Figure 10). Thus, the increase in stability in serum results from the 2'-substitutions.

**EXAMPLE 7. EFFICACY OF NX31975-40K PEG (SEQ ID NO:146) IN RESTENOSIS**

Rat Restenosis Model and Efficacy Results. The plasma residence time of Nucleic Acid Ligands is dramatically improved by the addition of large, inert functional groups such as polyethylene glycol (see for example PCT/US 97/18944). For *in vivo* efficacy experiments, 40K PEG was conjugated to NX31975 to create NX31975-40K PEG as described in Example 5B (see Figure 9A for molecular description). Importantly, based on binding experiments, the addition of 40 kDa PEG group at the 5'-end of the ligand does not affect its binding affinity for PDGF-BB.

The effect of selective inhibition of PDGF-B by NX31975-40K PEG was studied in three-month-old male Sprague-Dawley rats (370-450 g). The rats were housed three to a cage with free access to a standard laboratory diet and water. Artificial light was provided 14 hours per day. The experiments were performed in accordance with the institutional guidelines at the Animal Department, Department of Surgery, University Hospital, Uppsala University, Sweden.

A total of 30 rats were randomly allocated to one of two treatment groups: 15 rats in group one received 10 mg/kg body weight of NX31975-40K PEG in phosphate buffered saline (PBS) twice daily delivered by intraperitoneal (i.p.) injections and 15 rats in group two (the control group) received an equal volume of PBS (about 1 ml). The duration of treatment was 14 days. The first injections in both groups were given one hour before arterial injury.

To generate the arterial lesions, all animals were anaesthetized with an i.p. injection of a mixture of one part Fentanyl-fluanisone (Hypnorm vet, fluanisone 10 mg/ml, fentanyl 0.2 mg/ml, Janssen Pharmaceutica, Beerse, Belgium), one part midazolam (Dormicum, Midazolam 5 mg/ml. F. Hoffman-La Roche AG, Basel, Switzerland) and two parts sterile water, 0.33 ml/100 g rat. The distal left common carotid and external carotid arteries were exposed through a midline incision in the neck. The left common carotid artery was traumatized by intraluminal passage of 2F Fogarty embolectomy catheter introduced through the external carotid artery. The catheter was passed three times with the balloon expanded sufficiently with 0.06 ml distilled water to achieve a distension of the carotid itself. The external carotid was ligated after removal of the catheter and the wound was closed. All surgical procedures were performed by a surgeon blinded to the treatment groups.

Fourteen days after the catheter injury, the animals were anesthetized as above. Twenty minutes before the exposure of the abdominal aorta the animals received an intravenous injection of 0.5 ml 0.5% Evans blue dye (Sigma Chemical Co., St. Louis, MO) to allow identification of the vessel segment which remained deendothelialized. The carotid arteries were perfused with ice-chilled PBS *in situ* at 100 mm Hg, via a large cannula placed retrograde in the abdominal aorta until the effluent ran clear via inferior caval vein vent. A distal half of the right and left common carotid arteries, up to the level of the bifurcation, were removed and frozen in liquid nitrogen. Immediately thereafter, the remaining

proximal segment was perfusion-fixed through the same aortic cannula at 100 mm Hg pressure with 2.5% glutaraldehyde in phosphate buffer, pH 7.3. Before starting perfusion with PBS, the animals were killed by an overdose of phenobarbital. After approximately 15 minutes of perfusion fixation, the remaining proximal right and left common carotid arteries were retrieved for further preparation, including the aortic arch and innominate artery.

Five sections, approximately 0.5  $\mu$ m apart, from the middle of the Evans blue stained segment of the left common carotid artery and one section from the contralateral uninjured artery were analyzed per animal with computer-assisted planimetry. The following areas were measured: the area encircled by external elastic lumina (EEL), internal elastic lumina (IEL) and the endoluminal cell layer. Areas for tunical media and tunica intima were calculated. All measurements by an individual blinded to the treatment regimens.

Based on values of intima/media ratios for the control and the Nucleic Acid Ligand-treated groups, the PDGF Nucleic Acid Ligand significantly ( $p < 0.05$ ) inhibited about 50% of the neointima formation (Figure 11).

#### **EXAMPLE 8. ANTAGONISM OF PDGF IN GLOMERULONEPHRITIS BY NX31975-40K PEG**

This example provides the general procedures followed and incorporated in Example 9.

##### **Materials and Methods**

All Nucleic Acid Ligands and their sequence-scrambled controls were synthesized by the solid phase phosphoramidite method on controlled pore glass using an 8800 Milligen DNA Synthesizer and deprotected using ammonium hydroxide at 55°C for 16 h. The Nucleic Acid Ligand used in experiments described in this example and Example 9 is NX31975-40K PEG (SEQ ID NO:146) (Figure 9A). NX31975-40K PEG was created by conjugating NX31975 (SEQ ID NO:148) (Table 7) to 40K PEG as described in Example 5. In the sequence-scrambled control Nucleic Acid Ligand, eight nucleotides in the helix junction region of NX31975 were interchanged without formally changing the consensus secondary structure (see Figure 8C). The binding affinity of the sequence-scrambled control Nucleic Acid Ligand for PDGF-BB is  $\sim 1 \mu$ M, which is 10,000 fold lower compared to NX21617 (SEQ ID NO:143). The sequence-scrambled control Nucleic Acid Ligand was

then conjugated to PEG and named NX31976-40K PEG (SEQ ID NO:147) (see Figure 9B for molecular description). The covalent coupling of PEG to the Nucleic Acid Ligand (or to the sequence-scrambled control) was accomplished as described in Example 5.

Rat PDGF-BB for cross-reactivity binding experiments was derived from *E. coli* transfected with sCR-Script Amp SK(+) plasmid containing the rat PDGF-BB sequence. Rat PDGF-BB sequence was derived rat lung poly A+ RNA (Clontech, San Diego, CA) through RT-PCR using primers that amplify sequence encoding the mature form of PDGF-BB. Rat PDGF-BB protein expression and purification was performed at R&D Systems.

## 10 Mesangial Cell Culture Experiments

Human mesangial cells were established in culture, characterized and maintained as described previously (Radeke *et al.* (1994) *J. Immunol.* 153:1281-1292). To examine the antiproliferative effect of the ligands on the cultured mesangial cells, cells were seeded in 96-well plates (Nunc, Wiesbaden, Germany) and grown to subconfluency. They were then growth-arrested for 48 hours in MCDB 302 medium (Sigma, Deisenhofen, Germany). After 48 hours various stimuli together with either 50 or 10 µg/ml Nucleic Acid Ligand NX31975-40K PEG or 50 or 10 µg/ml sequence-scrambled Nucleic Acid Ligand (NX31976-40K PEG) were added: medium alone, 100 ng/ml human recombinant PDGF-AA, -AB or -BB (kindly provided by J. Hoppe, University of Würzburg, Germany), 100 ng/ml human recombinant epidermal growth factor (EGF; Calbiochem, Bad Soden, Germany) or 100 ng/ml recombinant human fibroblast growth factor-2 (kindly provided by Synergen, Boulder, Colorado). Following 72 hours of incubation, numbers of viable cells were determined using 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT; Sigma) as described (Lonnemann *et al.* (1995) *Kidney Int.* 51:837-844).

## Experimental Design

Anti-Thy 1.1 mesangial proliferative glomerulonephritis was induced in 33 male Wistar rats (Charles River, Sulzfeld, Germany) weighing 150-160 g by injection of 1 mg/kg

monoclonal anti-Thy 1.1 antibody (clone OX-7; European Collection of Animal Cell Cultures, Salisbury, England). Rats were treated with Nucleic Acid Ligands or PEG (see below) from day 3 to 8 after disease induction. Treatment consisted of twice daily i.v. bolus injections of the substances dissolved in 400 µl PBS, pH 7.4. The treatment duration was

5 chosen to treat rats from about one day after the onset to the peak of mesangial cell proliferation (Floege *et al.* (1993) *Kidney Int. Suppl.* 39:S47-54). Four groups of rats were studied: 1) nine rats, who received NX31975-40K PEG (SEQ ID NO:146) (i.e., a total of 4 mg of the PDGF-B ligand coupled to 15.7 mg 40K PEG); 2) ten rats, who received an equivalent amount of PEG-coupled, scrambled Nucleic Acid Ligand (NX31976-40K PEG)

10 (SEQ ID NO:147); 3) eight rats, who received an equivalent amount (15.7 mg) of 40K PEG alone; 4) six rats, who received 400 µl bolus injections of PBS alone. Renal biopsies for histological evaluation were obtained on days 6 and 9 after disease induction. Twenty-four hour urine collections were performed from days 5 to 6 and 8 to 9 after disease induction. The thymidine analogue 5-bromo-2'-deoxyuridine (BrdU; Sigma, Deisenhofen, Germany;

15 100 mg/kg body weight) was injected intraperitoneally at 4 hours prior to sacrifice on day 9.

Normal ranges of proteinuria and renal histological parameter (see below) were established in 10 non-manipulated Wistar rats of similar age.

### Renal Morphology

20 Tissue for light microscopy and immunoperoxidase staining was fixed in methyl Carnoy's solution (Johnson *et al.* (1990) *Am. J. Pathol.* 136:369-374) and embedded in paraffin. Four µm sections were stained with the periodic acid Schiff (PAS) reagent and counterstained with hematoxylin. In the PAS stained sections the number of mitoses within 100 glomerular tufts was determined.

25

### Immunoperoxidase Staining

Four mm sections of methyl Carnoy's fixed biopsy tissue were processed by an indirect immunoperoxidase technique as described (Johnson *et al.* (1990) *Am. J. Pathol.* 136:369-374). Primary antibodies were identical to those described previously (Burg *et al.*

(1997) Lab. Invest. 76:505-516; Yoshimura *et al.* (1991) Kidney Int. 40:470-476) and included a murine monoclonal antibody (clone 1A4) to  $\alpha$ -smooth muscle actin; a murine monoclonal antibody (clone PGF-007) to PDGF B-chain; a murine monoclonal IgG antibody (clone ED1) to a cytoplasmic antigen present in monocytes, macrophages and dendritic cells; affinity purified polyclonal goat anti-human/bovine type IV collagen IgG preabsorbed with rat erythrocytes; an affinity purified IgG fraction of a polyclonal rabbit anti-rat fibronectin antibody; plus appropriate negative controls as described previously (Burg *et al.* (1997) Lab. Invest. 76:505-516; Yoshimura *et al.* (1991) Kidney Int. 40:470-476). Evaluation of all slides was performed by an observer, who was unaware of the origin of the slides.

To obtain mean numbers of infiltrating leukocytes in glomeruli, more than 50 consecutive cross sections of glomeruli containing more than 20 discrete capillary segments were evaluated and mean values per kidney were calculated. For the evaluation of the immunoperoxidase stains for  $\alpha$ -smooth muscle actin, PDGF B-chain, type IV collagen and fibronectin each glomerular area was graded semiquantitatively, and the mean score per biopsy was calculated. Each score reflects mainly changes in the extent rather than intensity of staining and depends on the percentage of the glomerular tuft area showing focally enhanced positive staining: I = 0-25%, II = 25-50%, III = 50-75%, IV = >75%. This semiquantitative scoring system is reproducible among different observers and the data are highly correlated with those obtained by computerized morphometry (Kliem *et al.* (1996) Kidney Int. 49:666-678; Hugo *et al.* (1996) J. Clin. Invest. 97:2499-2508).

### **Immunohistochemical Double-Staining**

Double immunostaining for the identification of the type of proliferating cells was performed as reported previously. (Kliem *et al.* (1996) Kidney Int. 49:666-678; Hugo *et al.* (1996) J. Clin. Invest. 97:2499-2508) by first staining the sections for proliferating cells with a murine monoclonal antibody (clone BU-1) against bromo-deoxyuridine containing nuclease in Tris buffered saline (Amersham, Braunschweig, Germany) using an indirect immunoperoxidase procedure. Sections were then incubated with the IgG<sub>1</sub> monoclonal



antibodies 1A4 against  $\alpha$ -smooth muscle actin and ED1 against monocytes/macrophages. Cells were identified as proliferating mesangial cells or monocytes/macrophages if they showed positive nuclear staining for BrdU and if the nucleus was completely surrounded by cytoplasm positive for  $\alpha$ -smooth muscle actin. Negative controls included omission of either of the primary antibodies in which case no double-staining was noted.

#### ***In situ* Hybridization for Type IV Collagen mRNA**

*In situ* hybridization was performed on 4 mm sections of biopsy tissue fixed in buffered 10% formalin utilizing a digoxigenin-labelled anti-sense RNA probe for type IV collagen (Eitner *et al.* (1997) *Kidney Int.* 51:69-78) as described (Yoshimura *et al.* (1991) *Kidney Int.* 40:470-476). Detection of the RNA probe was performed with an alkaline phosphatase coupled anti-digoxigenin antibody (Genius Nonradioactive Nucleic Acid Detection Kit, Boehringer-Mannheim, Mannheim, Germany) with subsequent color development. Controls consisted of hybridization with a sense probe to matched serial sections, by hybridization of the anti-sense probe to tissue sections which had been incubated with RNase A before hybridization, or by deletion of the probe, antibody or color solution described (Yoshimura *et al.* (1991) *Kidney Int.* 40:470-476). Glomerular mRNA expression was semiquantitatively assessed using the scoring system described above.

#### **Miscellaneous Measurements**

Urinary protein was measured using the Bio-Rad Protein Assay (Bio-Rad Laboratories GmbH, München, Germany) and bovine serum albumin (Sigma) as a standard.

#### **Statistical Analysis**

All values are expressed as means  $\pm$  SD. Statistical significance (defined as  $p < 0.05$ ) was evaluated using ANOVA and Bonferroni t-tests.

#### **EXAMPLE 9.**

For all experiments reported here, the modified DNA Nucleic Acid Ligand was conjugated to 40K PEG as described in Examples 5 and 8 and shown in Figures 9A and 9B.

Since most Nucleic Acid Ligands have molecular weights ranging between 8 to 12 kDa (the modified PDGF Nucleic Acid Ligand has MW of 10 kDa), the addition of a large inert molecular entity such as PEG dramatically improves the residence times of Nucleic Acid Ligands *in vivo* (see for example PCT/US 97/18944). Importantly, the addition of the PEG moiety to the 5' end of the Nucleic Acid Ligand has no effect on the binding affinity of the Nucleic Acid Ligand for PDGF-BB ( $K_d \sim 1 \times 10^{-10}$  M).

### Cross-reactivity of Nucleic Acid Ligands for Rat PDGF-BB

The sequence of PDGF is highly conserved among species, and human and rat PDGF B-chain sequences are 89% identical (Herren *et al.* (1993) *Biochim. Biophys. Acta* 1173:294; Lindner *et al.* (1995) *Circ. Res.* 76:951). Nevertheless, in view of the high specificity of Nucleic Acid Ligands (Gold *et al.* (1995) *Ann. Rev. Biochem.* 64:763-797), the correct interpretation of the *in vivo* experiments requires understanding of the binding properties of the Nucleic Acid Ligands to rat PDGF B-chain. We have therefore cloned and expressed the mature form of rat PDGF-BB in *E. coli*. The PDGF Nucleic Acid Ligands bound to rat and human recombinant PDGF-BB with the same high affinity (data not shown).

### PDGF B-Chain DNA-Ligand Specifically Inhibits Mesangial Cell Proliferation *in vitro*

In growth arrested mesangial cells, the effects of NX31975-40K PEG (SEQ ID NO:146) or the scrambled Nucleic Acid Ligand (NX31976-40K PEG) (SEQ ID NO:147) on growth factor induced proliferation were tested. Stimulated growth rates of the cells were not affected by the addition of scrambled Nucleic Acid Ligand (Figure 12). Fifty  $\mu$ g/ml of NX31975-40K PEG significantly reduced PDGF-BB induced mesangial cell growth (Figure 12). PDGF-AB and -AA induced mesangial cell growth also tended to be lower with NX31975-40K PEG, but these differences failed to reach statistical significance (Figure 12). In contrast, no effects of NX31975-40K PEG on either EGF or FGF-2 induced growth were noted. Similar effects were noted if the Nucleic Acid Ligands were used at a concentration of 10  $\mu$ g/ml (data not shown).

### Effects of PDGF B-Chain DNA-Ligand in Rats with Anti-Thy 1.1 Nephritis

Following the injection of anti-Thy 1.1 antibody, PBS treated animals developed the typical course of the nephritis, which is characterized by early mesangiolysis and followed by a phase of mesangial cell proliferation and matrix accumulation on days 6 and 9 (Floege  
5 *et al.* (1993) *Kidney Int. Suppl.* 39:S47-54). No obvious adverse effects were noted following the repeated injection of Nucleic Acid Ligands or PEG alone, and all rats survived and appeared normal until the end of the study.

In PAS stained renal sections the mesangioproliferative changes on days 6 and 9 after disease induction were severe and indistinguishable among rats receiving PBS, PEG  
10 alone or the scrambled Nucleic Acid Ligand (data not shown). Histological changes were markedly reduced and almost normalized in the NX31975-40K PEG ligand treated group. In order to (semi-)quantitatively evaluate the mesangioproliferative changes, various parameters were analyzed:

#### 15 a) Reduction of Mesangial Cell Proliferation

Glomerular cell proliferation, as assessed by counting the number of glomerular mitoses, was not significantly different between the three control groups on days 6 and 9 (Figure 13A). As compared to rats receiving the scrambled Nucleic Acid Ligand, treatment with PDGF-B ligand led to a reduction of glomerular mitoses by 64% on day 6 and by 78%  
20 on day 9 (Figure 13A). To assess the treatment effects on mesangial cells, the renal sections for  $\alpha$ -smooth muscle actin were immunostained, which is expressed by activated mesangial cells only (Johnson *et al.* (1991) *J. Clin. Invest.* 87:847-858). Again, there were no significant differences between the three control groups on days 6 and 9. However, the immunostaining scores of  $\alpha$ -smooth muscle actin were significantly reduced on day 6 and 9  
25 in the NX31975-40K PEG treated group (Figure 13D). To specifically determine whether mesangial cell proliferation was reduced, NX31975-40K PEG treated rats and scrambled Nucleic Acid Ligand treated rats were double immunostained for a cell proliferation marker (BrdU) and  $\alpha$ -smooth muscle actin. The data confirmed a marked decrease of proliferating mesangial cells on day 9 after disease induction:  $2.2 \pm 0.8$  BrdU-/ $\alpha$ -smooth muscle actin

positive cells per glomerular cross section in PDGF-B aptamer treated rats versus  $43.3 \pm 12.4$  cells in rats receiving the scrambled Nucleic Acid Ligand, i.e., a 95% reduction of mesangial cell proliferation. In contrast, no effect of the PDGF-B aptamer was noted on proliferating monocytes/macrophages on day 9 after disease induction (PDGF-B aptamer treated rates:  $2.8 \pm 1.1$  BrdU+/ED-1+ cells per 100 glomerular cross sections; scrambled aptamer treated rats:  $2.7 \pm 1.8$ ).

#### **b) Reduced Expression of Endogenous PDGF B-Chain**

By immunohistochemistry, the glomerular PDGF B-chain expression was markedly upregulated in all three control groups (Figure 13B), similar to previous observations (Yoshimura *et al.* (1991) *Kidney Int.* 40:470-476). In the NX31975-40K PEG treated group the glomerular overexpression of PDGF B-chain was significantly reduced in parallel with the reduction of proliferating mesangial cells (Figure 13B).

#### **c) Reduction of Glomerular Monocyte/Macrophage Influx**

The glomerular monocyte/macrophage influx was significantly reduced in the NX31975-40K PEG treated rats as compared to rats receiving scrambled Nucleic Acid Ligand on days 6 and 9 after disease induction (Figure 13E).

#### **d) Effects on Proteinuria**

Moderate proteinuria of up to 147 mg/24 hrs was present on day 6 after disease induction in the 3 control groups (Figure 13C). Treatment with NX31975-40K PEG reduced the mean proteinuria on day 6, but this failed to reach statistical significance (Figure 13C). Proteinuria on day 9 after disease induction was low and similar in all four groups (Figure 13C).

#### **e) Reduction of Glomerular Matrix Production and Accumulation**

By immunohistochemistry, marked glomerular accumulation of type IV collagen and fibronectin was noted in all three control groups (Figures 14A-C). The overexpression

of both glomerular type IV collagen and fibronectin was significantly reduced in NX31975-40K PEG treated rats (Figures 14A-C). In the latter, glomerular staining scores approached those observed in normal rats (Figures 14A-C). By *in situ* hybridization, the decreased glomerular expression of type IV collagen in NX31975-40K PEG treated rats was shown to be associated with decreased glomerular synthesis of this collagen type (Figures 14A-C).

**EXAMPLE 10. EXPERIMENTAL PROCEDURE FOR EVOLVING  
2'-FLUORO-2'-DEOXYPYRIMIDINE RNA LIGANDS TO PDGF AND RNA  
SEQUENCES OBTAINED.**

**2'-FLUORO-2'-DEOXYPYRIMIDINE RNA SELEX**

SELEX with 2'-fluoro-2'-deoxypyrimidine RNA targeting PDGF-AB was done essentially as described previously (*vide supra*, and Jellinek *et al.*, 1993, 1994: *supra*) using the primer template set as shown in Table 8 (SEQ ID NOS:36-38). Briefly, the 2'-fluoro-2'-deoxypyrimidine RNA for affinity selections was prepared by *in vitro* transcription from synthetic DNA templates using T7 RNA polymerase (Milligan *et al.* (1987) Nucl. Acids Res. 15:8783). The conditions for *in vitro* transcription described in detail previously (Jellinek *et al.* (1994) *supra*) were used, except that higher concentration (3 mM) of the 2'-fluoro-2'-deoxypyrimidine nucleoside triphosphates (2'-F-UTP and 2'-F-CTP) was used compared to ATP and GTP (1 mM). Affinity selections were done by incubating PDGF-AB with 2'-fluoro-2'-deoxypyrimidine RNA for at least 15 min at 37°C in PBS containing 0.01% human serum albumin. Partitioning of free RNA from protein-bound RNA was done by nitrocellulose filtration as described (Jellinek *et al.*, 1993, 1994: *supra*). Reverse transcription of the affinity-selected RNA and amplification by PCR were done as described previously (Jellinek *et al.* (1994) *supra*). Nineteen rounds of SELEX were performed, typically selecting between 1-12% of the input RNA. For the first eight rounds of selection, suramin (3-15  $\mu$ M) was included in the selection buffer to increase the selection pressure. The affinity-enriched pool (round 19) was cloned and sequenced as described (Schneider *et al.* (1992) *supra*). Forty-six unique sequences have been identified, and the sequences are shown in Table 9 (SEQ ID NOS:39-81). The unique-sequence ligands were screened for their ability to bind PDGF-AB with high affinity. While random 2'-fluoropyrimidine RNA (Table 8) bound to PDGF with a dissociation constant (Kd) of  $35 \pm 7$  nM, many of the

affinity-selected ligands bound to PDGF-AB with  $\approx 100$ -fold higher affinities. Among the unique ligands, clones 9 ( $K_d = 91 \pm 16$  pM), 11 ( $K_d = 120 \pm 21$  pM), 16 ( $K_d = 116 \pm 34$  pM), 23 ( $K_d = 173 \pm 38$  pM), 25 ( $K_d = 80 \pm 22$  pM), 37 ( $K_d = 97 \pm 29$  pM), ( $K_d = 74 \pm 39$  pM), and 40 ( $K_d = 91 \pm 32$  pM) exhibited the highest affinity for PDGF-AB (binding of all  
5 of these ligands to PDGF-AB is biphasic and the  $K_d$  for the higher affinity binding component is given).

**Table 1.** Starting DNA and PCR primers for the ssDNA SELEX experiment.

	SEQ ID NO:
Starting ssDNA:	
5'-ATCCGCCTGATTAGCGATACT[-40N-]ACTTGAGCAAAATCACCTGCAGGGG-3'	1
PCR Primer 3N2*:	
5'-BBBCCCCTGCAGGTGATTTTGCTCAAGT-3'	2
5 PCR Primer 5N2**:	
5'-CCGAAGCTTAATACGACTCACTATAGGG <u>ATCCGCCTGATTAGCGATACT</u> -3'	3

\*B=biotin phosphoramidite (e. g., Glen Research, Sterling, VA)

\*\*For rounds 10, 11, and 12, the truncated PCR primer 5N2 (underlined) was used to amplify the template.

**Table 2.** Unique Sequences of the ssDNA high affinity ligands to PDGF.

5'-ATCCGCCTGATTAGCGATACT [40N] ACTTGAGCAAAATCACCTGCAGGGG-3'		SEQ ID NO:
5	*14	4
	*4/	5
	6	6
10	23	7
	2	8
	34	9
	8	10
	1	11
15	5	12
	*40	13
	47	14
	18	15
	30	16
20	*20	17
	35	18
	13	19
	16	20
	*36	21
25	50	22
	4	23
	44	24
	24	25
	7	26
30	*26	27
	19	28
	48	29
	46	30
	25	31
35	31	32
	12	33
	15	34
	*38	35

40 \* Indicates a boundary experiment was performed.

Italics indicate the clones that were found to retain high affinity binding as minimal ligands.



Table 3.

		HELIX I		
		HELIX II	HELIX III	
SEQ ID NO: Group A				
8	2	=AGGG--AGGA--TACG-----TCTG-AGC-ATCac3' 5'ATGTGAT-CCCTGCAG=		
23	4	ACCGGG--CTAC--TTC-----GTAG-AGC-ATC-----TCT-----GAT-CCCGGTGCTCG		
26	7	GGG--CGACC-TTCT-----GGACG-AGC-ATCAC--CTAT--GTGAT-CCCG		
20	16	CLGAGG--CATG--TTAA-----CATG-AGC-ATCGT--CTC---ACGAT-CTCAGCC		
21	36	CCACAGG--CTACG-GCA-----CGTAG-AGC-ATCA---CCA---TGAT-CTGTG		
35	38	AAAGTCGTGCAGGG--TCC--CCT-----GGA-AGC-ATC-----TCC-----GAT-CCCAGactt		
24	44	AAAGGG--CGAAC-GTA-----GGTCG-AGGCATCC--ATT---GGAT-CCCTTC		
22	50	GCGGG--CATG--GCA-----CATG-AGC-ATC-----TCT-----GAT-CCCGCAATCCTC		
7	23	=AGG--CAGGATAAC--GTCCCTG-AGC-ATCac3' 5'AGGTGATCCCTGCAA=		
9	34	=GGG--CTGC--GCAAAATA--GCAG-AGC-ATCac3' 5'CACGTGAT-CCCATAA=		
SEQ ID NO: Group B				
19	13	GCTCGTAGG--GGGCGA--TTCTT-----TCGCC-GTT-ACT-----TCC-----AGT-CTTAc		
4	14	tactAGG--CTT--GACA-----AAG-GGC-ACCAT--GGCTTAGTGGT-CTTAGTa		
34	15	cTCAAGTAGGG--CGGAC-ACAC-----GTCCG-GGC-ACC---TAA-----GGT-CCCacttgag		
15	18	cLGGG--CGCCCTAAACAA--AGGGTG-GTC-ACT-----TCT-----AGT-CCCAGGA		
17	20	ATGGGAGGG--CGCG--TTCTT-----CGTG-GTT-ACT-----TTT-----AGT-CCCG		
31	25	cLTTGGG--CGTG--AATGTC-----CACG-GGT-ACC---TCC-----GGT-CCCAAGAG		
16	30	TCCGGG--CTCGG-GAT-----TCGTG-GTC-ACT-----TTC-----AGT-CCCGGATATA		
32	31	=AGGG--CAG--CCCTAA-----CTG-GTC-acttgagc3' 5'TCCGCGCAAGT-CCCTGGTAA=		
18	35	ACGGGAGGG--CACG--TTCTT-----CGTG-GTT-ACT-----TTT-----AGT-CCCG		
5	41	=GGG--CTGAGTa3' 5'tactCAG-GGC-actGCAAGCAATTGTGGT-CCCAAT=		
14	47	GTGGGTGGGATCGGG--ATG-----CCTC-GTC-ACT-----TCT-----AGT-CCact		

**Table 4.** Frequency of base pairs in the helical regions of the consensus motif shown in **Figure 1.**

		Base pair <sup>b</sup>						
	<u>Position<sup>a</sup></u>	<u>AT</u>	<u>TA</u>	<u>GC</u>	<u>CG</u>	<u>TG</u>	<u>GT</u>	<u>other</u>
5	I-1	0	0	21	0	0	0	0
	I-2	0	0	21	0	0	0	0
	I-3	5	0	16	0	0	0	0
	I-4	3	5	1	4	1	0	7
	I-5	2	3	3	4	0	0	9
15	II-1	0	1	2	17	0	0	1
	II-2	5	5	5	1	0	4	1
	II-3	3	4	7	6	0	0	1
	II-4	3	0	8	5	0	0	4
20	III-1	21	0	0	0	0	0	0
	III-2	0	10	0	11	0	0	0
	III-3	0	7	0	13	1	0	0

<sup>a</sup> Helices are numbered with Roman numerals as shown in Figure 1. Individual base pairs are numbered with Arabic numerals starting with position 1 at the helix junction and increasing with increased distance from the junction.

<sup>b</sup> The TG and GT base pairs to the Watson-Crick base pairs for this analysis were included. There is a total of 21 sequences in the set.

**Table 5.** Affinities of the minimal DNA ligands to PDGF-AA, PDGF-AB and PDGF-BB.

	<u>Ligand</u>	<u>K<sub>d</sub>, nM</u>		
		<u>PDGF AA<sup>a</sup></u>	<u>PDGF-AB<sup>b</sup></u>	<u>PDGF BB<sup>b</sup></u>
5	20t	47 ± 4	0.147 ± 0.011	0.127 ± 0.031
	36t	72 ± 12	0.094 ± 0.011	0.093 ± 0.009
10	41t	49 ± 8	0.138 ± 0.009	0.129 ± 0.011

<sup>a</sup>Data points shown in Figure 3A were fitted to equation (1) (Example 1).

15 <sup>b</sup>Data points in Figures 3B and 3C were fitted to equation (2). The dissociation constant (K<sub>d</sub>) values shown are for the higher affinity binding component. The mole fraction of DNA that binds to PDGF-AB or PDGF-BB as the high affinity component ranges between 0.58 to 0.88. The K<sub>d</sub> values for the lower affinity interaction range between 13 to 78 nM.

**Table 6.** Relative affinity for PDGF-AB of ligand 36t variants.

		SEQ		Composition*	$K_d^{\text{ligand}}/K_d^{36t}$ **
Ligand	ID NO:				
5	36t	84	CACAGGCTACGGCACGTAGAGCATCACCATGATCCTGTG[ <sup>3'</sup> T]		1.0
	1073	97	<u>CACAGGCTACGGCACGTAGAGCATCACCATGATCCTGUG</u> [ <sup>3'</sup> T]		11.8
	1074	98	CACAGGCTAC <u>GGCACGU</u> AGAGCATCACCATGATCCTGTG[ <sup>3'</sup> T]		3.1
	1075	99	CACAGGCTACGGCACGTAGAGCATC <u>ACCAUG</u> ATCCTGTG[ <sup>3'</sup> T]		10
	1076	100	<u>CACAGGCTACGGCACGU</u> AGAGCATC <u>ACCAUG</u> ATCCTGTG[ <sup>3'</sup> T]		440
10	1145	101	CACAGGCTACGGCACGTAGAGCATC <u>ACCATG</u> ATCCTGTG[ <sup>3'</sup> T]		0.27
	1148	102	CAC <u>AGGCU</u> ACGGCACGTAGAGCATCACCATGATCCTGTG[ <sup>3'</sup> T]		281
	1144	103	CACAGGCTACGGCACGTAGAGCATCACCAT <u>GAUCCUG</u> TG[ <sup>3'</sup> T]		994
	1142	104	CACAGGCTACGGCACG <u>U</u> AGAGCA <u>U</u> CACCATGATCCTGTG[ <sup>3'</sup> T]		12.9
	1149	105	CACAGGCTACGGCACGTAG <u>AGCAT</u> CACCATGATCCTGTG[ <sup>3'</sup> T]		2.9
15	EV1	106	CACAGGCTACGGCACGTAG <u>AGCAT</u> CACCATGATCCTGTG[ <sup>3'</sup> T]		35.1
	EV2	107	CACAGGCTACGG <u>CACGU</u> AGAGCATCACCATGATCCTGTG[ <sup>3'</sup> T]		5.3
	EV3	108	CACAGGCTAC <u>GGCAC</u> GTAGAGCATCACCATGATCCTGTG[ <sup>3'</sup> T]		1.5
	EV4	109	<u>CACAGGCTACGGCACGTAGAGCATCACCATGATCCTGTG</u> [ <sup>3'</sup> T]		4.5
	EV5	110	CACAGGCTACGGCACGTAGAGCATCACCATGATCC <u>UGUG</u> [ <sup>3'</sup> T]		2.3
20	1157	111	CACAGGCTACGGCACGTAGAGCATCACCATGATCCTGTG[ <sup>3'</sup> T]		1.0
	1160	112	CACAGGCTACGGCACGTAGAGCATCACCATGATCCTGTG[ <sup>3'</sup> T]		1.4
	1161	113	CACAGGCTACGGCACGTAGAGCATCACCATGATCCTGTG[ <sup>3'</sup> T]		0.22
	1162	114	CACAGGCTACGGCACGTAGAGCATCACCATGATCCTGTG[ <sup>3'</sup> T]		0.52
	1165	115	CACAGGCTACGGCACGTAGAGCATCACCATGATCCTGTG[ <sup>3'</sup> T]		0.61
25	1164	116	CACAGGCUACGGCACGTAGAGCATCACCATGATCCTGTG[ <sup>3'</sup> T]		0.45
	1166	117	CACAGGCTACGGCACG <u>U</u> AGAGCATCACCATGATCCTGTG[ <sup>3'</sup> T]		0.76
	1159	118	CACAGGCTACGGCACGTAGAGCA <u>U</u> CACCATGATCCTGTG[ <sup>3'</sup> T]		0.37
	1163	119	CACAGGCTACGGCACGTAGAGCATC <u>ACCAUG</u> ATCCTGTG[ <sup>3'</sup> T]		1.3
	1158	120	CACAGGCTACGGCACGTAGAGCATCACCATGA <u>U</u> CCTGTG[ <sup>3'</sup> T]		2.4
30	1255	121	CACAGGCUACGGCACG <u>U</u> AGAGCA <u>U</u> CACCATGA <u>UCCUG</u> TG[ <sup>3'</sup> T]		24.2
	1257	122	CACAGGCTAC <u>GGCAC</u> GTAGAGCATC <u>ACCATG</u> ATCCTGUG[ <sup>3'</sup> T]		1.3
	1265	123	CACAGGCTAC <u>GGCAC</u> GTAGAGCATC <u>ACCATG</u> ATCCUGUG[ <sup>3'</sup> T]		1.4
	1266	124	CACAGGCUAC <u>GGCAC</u> GTAGAGCA <u>U</u> CACCATGATCCUGUG[ <sup>3'</sup> T]		1.0
	1267	125	CACAGGCTAC <u>GGCAC</u> GTAGAGCATC <u>ACCATG</u> ATCCUGUG[ <sup>3'</sup> T]		4.2
35	1269	126	CACAGGCUAC <u>GGCAC</u> GTAGAGCATC <u>ACCATG</u> ATCCUGUG[ <sup>3'</sup> T]		0.87
	1295	127	CAGGCUAC <u>GGCAC</u> GTAGAGCA <u>U</u> CACCATGATCCUG[ <sup>3'</sup> T]		0.9
	1296	128	CAGGCU- <u>CGGCACG</u> -AGAGCA <u>U</u> CACCATGATCCUG[ <sup>3'</sup> T]		2.1
	1297	129	CAGGCU- <u>CGGCACG</u> -AGAGCA <u>U</u> C-CCA-GATCCUG[ <sup>3'</sup> T]		2.9
	1303	130	CAGGCUAC <u>GGCAC</u> GTAGAGCA <u>U</u> CACCATGATCCUG[ <sup>3'</sup> T]		5.8
40	1304	131	CAGGCUAC <u>GGCAC</u> GTAGAGCA <u>U</u> CACCATGATCCUG[ <sup>3'</sup> T]		607
	1305	132	CAGGCUAC <u>GGCAC</u> GTAGAGCA <u>U</u> CACCATGATCCUG[ <sup>3'</sup> T]		196
	1306	133	CAGGCUAC <u>GGCAC</u> GTAGAGCA <u>U</u> CACCATGATCCUG[ <sup>3'</sup> T]		4.4
	1327	134	CAGGCUAC <u>GGCAC</u> GTAGAGCA <u>U</u> CACCATGATCCUG[ <sup>3'</sup> T]		0.63

	SEQ		Composition*	$K_d^{\text{ligand}} / K_d^{36t} **$
	Ligand	ID NO:		
	1328	135	CAGGCU <u>ACGGC</u> ACGTAGAGCAUC <u>ACCAT</u> GTATCCUG[ <sup>3'</sup> T]	2.2
	1329	136	CAGGCU <u>ACGGC</u> ACGTAGAGCAUC <u>ACCAT</u> GTATCCUG[ <sup>3'</sup> T]	0.72
5	1369	137	CAGGCU <u>ACGGC</u> ACGTAGAGCAUC <u>ACCAT</u> GTATCCUG[ <sup>3'</sup> T]	0.37
	1374	138	CAGGCU <u>ACGGC</u> ACGTAGAGCAUC <u>ACCAT</u> GTATCCUG[ <sup>3'</sup> T]	1.5
	1358	139	CAGGCU <u>ACG</u> -S-CGTAGAGCAUCA-S-TGATCCUG[ <sup>3'</sup> T]	0.54
	1441	140	CAGGCU <u>ACG</u> -S-CGTAGAGCAUCA-S-TGATCCUG[ <sup>3'</sup> T]	0.33

10

\*A,C,G,T=deoxy-A,C,G,T; A,C,G,U=2'-OMe-A,C,G,T; C,U=2'-fluoro-C,U; S=hexaethyleneglycol spacer; [3'T]=inverted (3'-3') T.

\*\* $K_d^{36t}$  value of 0.178±88 pM used for the calculation is average, with standard deviation, of four independent measurements (94±11, 161±24, 155±30 and 302±32 pM).

15

**Table 7.** Relative affinity for PDGF-BB of ligand 36ta variants.

The effect of various substitutions on the affinity of ligand 36ta for PDGF-BB.

5	<u>Ligand</u>	<u>Composition*</u>	$\frac{K_d^{\text{ligand}}}{K_d^{36ta}}$ **	SEQ
				ID NO:
	36ta	CAGGCTACGGCACGTAGAGCATCACCATGATCCTG[3'T]	1.0	141
	NX21568	CAGGCUACG-S-CGTAGAGCAUCA-S-TGATCCUG[3'T]	0.63	142
	NX21617	[L1]CAGGCUACG-S-CGTAGAGCAUCA-S-TGATCCUG[3'T]	0.54	143
10	NX21618	[L1]CAGGCUACG-S-CGTAGAGCAUCA-S-TGAUCCTG[3'T]	418	144
	NX31975	[L2]CAGGCUACG-S-CGTAGAGCAUCA-S-TGATCCUG[3'T]	0.54	148
	NX31976	[L2]CAGGCUACG-S-CGTACCGATUCA-S-TGAAGCU[3'T]		149

15 \*A,C,G,T=deoxy-A,C,G,T; A,G =2'-OMe-A,G; C,U=2'-F-C,U; S=hexaethyleneglycol spacer (from Spacer Phosphoramidite 18, Glen Research, Sterling, VA); [3'T]=inverted (3'-3') T; [L1]=dT amine (Glen Research, Sterling, VA); [L2] = pentyl amino linker.

\*\* $K_d^{36ta} = 0.159 \pm 13$  pM.

**Table 8.** Starting RNA and PCR primers for the 2'-fluoropyrimidine RNA SELEX experiment.

5		SEQ ID NO:
	Starting 2'-fluoropyrimidine RNA:	
	Starting RNA:	
10	5'-GGGAGACAAGAAUAACGCUCAA[-50 N-]UUCGACAGGAGGCUCACAACAGGC-3'	36
	PCR Primer 1:	
	5'-TAATACGACTCACTATAGGGAGACAAGAATAACGCTCAA-3'	37
	PCR Primer 2:	
15	5'-GCCTGTTGTGAGCCTCCTGTCGAA-3'	38

**Table 9.** Sequences of the evolved region of 2'-fluoropyrimidine RNA high affinity ligands to PDGF-AB. Sequences of the fixed region (Table 8) are not shown.

		SEQ ID NO:
5	1 CGGUGGCAUUUCUACUUCUUCUCGCUUUCUCGCGUUGGGCNCGA	39
	2 CCAACCUUCUGUCGGCGUUGCUUUUUGGACGGCACUCAGGCUCCA	40
	3 UCGAUCGGUUGUGUGCCGGACAGCCUUAACAGGGCUGGGACCGAGGCC	41
10	4 CUGAGUAGGGGAGGAAGUUGAAUCAGUUGUGGGCGCCUCUCAUUCGC	42
	5 CAGCACUUUCGCUUUUCAUCAUUUUUUCUUCACUGUUGGGCGCGGAA	43
	6 UCAGUGCUGGGGCUAUGUCUCGAUGGGGAUUUUUCUUCAGCACUUUGCCA	44
	7 UCUACUUUCCAUUUCUCUUUUCUUCACAGCGGGUUUCCAGUGAACCA	45
	8 CGAUAGUGACUACGAUGACGAAGGCCGCGGGUUGGAUGCCCGCAUUGA	46
15	10 GUCGAUACUGGGGACUUGCUCUUAUGGCCGAUUAACGAUUCGGUCAG	47
	13 GUGCAAACUUAACCCGGGAACCGCGCGUUUCGAUCGACUUUCCUUUCCA	48
	15 AUUCCGCGUUCGGAUUAUUCUGUGCUCGGAAAUCGGUAGCCAUAGUGCA	49
	16 CGAACGAGGAGGGAGUGGCAAGGGAUGGUUGGAUAGGCUCUACGCUCA	50
	17 GCGAAACUGGGGACUUGCUCUUAUGGCCGAUUAACGAUUCGGUUCU	51
20	18 CGAACGAGGAGGGAGUCGCAAGGGAUGGUUGGAUAGGCUCUACGCUCAA	52
	19 CGAGAAGUGACUACGAUGACGAAGGCCGCGGGUUGAAUCCCUCAUUGA	53
	20 AAGCAACGAGACCUGACGCCUGAUGUGACUGUGCUUGCACCCGAUUCUG	54
	21 GUGAUUCUCAUUCUCAUUGCUUUCACAACUUUUUCCACUUCAGCGUGA	55
	22 AAGCAACGAGACUCGACGCCUGAUGUGACUGUGCUUGCACCCGAUUCU	56
25	23 UCGAUCGGUUGUGUGCCGGACAGCUUUGACCAUGAGCUGGGACCGAGGCC	57
	24 NGACGNGUGGACCUGACUAAUCGACUGAUCAAAGAUCGCGCCAGAUUGG	58
	26 CACUGCGACUUGCAGAAGCCUUGUGUGGCGGUACCCCUUUGGCCUCG	59
	27 GGUGGCAUUUCUUCAUUUUCCUUCUCGCUUUCUCCGCCGUUGGGCGCG	60
	29 CCUGAGUAGGGGGGAAAGUUGAAUCAGUUGUGGCGCUCUACUCAUUCGCC	61
30	30 GUCGAAACUGGGGACUUGCUCUUAUGGCCGAUUAACGAUUCGGUUCA	62
	31 GCGAUACUGGGGACUUGCUCUUAUGGCCGAUUAACGAUUCGGCUCAG	63
	32 ACGUGGGGCACAGGACCGAGAGUCCUCCGGCAUAGCCGCUACCCACC	64
	33 CACAGCCUNANAGGGGGGAAGUUGAAUCAGUUGUGGCGCUCUACUCAUUCGC	65
	34 ANGGGUAUGGUGACUUGCUCUUAUGGCCGAUUAACGAUUCGGUCAG	66
35	35 CCUGCGUAGGGNGGGAAGUUGAAUCAGUUGUGGCGCUCUACUCAUUCGCC	67
	39 CGAACGAGGAGGGAGUGGCAAGGGAUGGUUGGAUAGGCUCUACGCUCA	68
	41 GUGCAAACUUAACCCGGGAACCGCGCGUUCGAUUCGCUUCCNUAUCCA	69
	42 CGAACGAGGAGGGAGUGGCAAGGGACGGUNNAUAGGCUCUACGCUCA	70
	43 UCGGUGUGGCUCAGAAACUGACACGCGUGAGCUUCGCACACAUCUGC	71
40	44 UAUCGCUUUUCAUCAAUUCACUUAUUUACUCUNUAACUUGGGCGUGCA	72
	45 GUGCAAACUUAACCCGGGAACCGCGCGUUCGAUCCUGCAUCCUUUUUCC	73
	46 UCGNUCGGUUGUGUGCCGGCAGCUUUGUCCAGCGUUGGGCCGAGGCC	74
	47 AGUACCAUCUCAUCUUAUUCUUAUUCUUAAGGCACAUAUGAGGGU	75
	49 CCUGAGUAGGGGGGGAAGUUGAACAGUUGUGGCGNCCUACUCAUUCNCCA	76
45	51 CCNNCCUNCUGUCGGCGCUUGUCUUUUUGGACGGGCAACCCAGGGCUC	77
	52 CCAACCUNCUGUCGGCGCUUGUCUUUUUGGACGAGCAACUCAAGGCUCGU	78
	53 CCAGCGCAGAUCCCGGGCUGAAGUGACUGCCGGCAACGGCCGCUCCA	79
	54 UUCCCGUAACAACUUUUAUUUUAUUUUAUCCAACAGUGAGCAGCA	80
	55 UAUCGCUUUAUCAAAUCCACUCCUACUUCUUUAACUUGGGCGUGCA	81



## SEQUENCE LISTING

- (1) GENERAL INFORMATION:
- (i) APPLICANT: NEBOJSA JANJIC, LARRY GOLD
- 5 (ii) TITLE OF THE INVENTION: PLATELET DERIVED GROWTH FACTOR (PDGF)  
NUCLEIC ACID LIGANDS
- (iii) NUMBER OF SEQUENCES: 149
- (iv) CORRESPONDENCE ADDRESS:
- 10 (A) ADDRESSEE: Swanson and Bratschun, L.L.C.  
(B) STREET: 8400 East Prentice Avenue, Suite #200  
(C) CITY: Denver  
(D) STATE: Colorado  
(E) COUNTRY: USA  
15 (F) ZIP: 80111
- (v) COMPUTER READABLE FORM:
- (A) MEDIUM TYPE: Diskette, 3.5 inch, 1.4 Mb storage  
(B) COMPUTER: IBM compatible  
(C) OPERATING SYSTEM: MS-DOS  
20 (D) SOFTWARE: WordPerfect 6.1
- (vi) CURRENT APPLICATION DATA:
- (A) APPLICATION NUMBER: PCT/US98/\_\_\_\_\_  
(B) FILING DATE:  
(C) CLASSIFICATION:
- 25 (vii) PRIOR APPLICATION DATA:  
(A) APPLICATION NUMBER: 08/991,743  
(B) FILING DATE: 16-DECEMBER-1997
- (vii) PRIOR APPLICATION DATA:
- (A) APPLICATION NUMBER: 08/618,693  
30 (B) FILING DATE: 20-MARCH-1996
- (vii) PRIOR APPLICATION DATA:
- (A) APPLICATION NUMBER: 08/479,783  
(B) FILING DATE: 7-JUNE-1995
- 35 (vii) PRIOR APPLICATION DATA:  
(A) APPLICATION NUMBER: 08/479,725  
(B) FILING DATE: 7-JUNE-1995
- (viii) ATTORNEY/AGENT INFORMATION:
- (A) NAME: Barry J. Swanson  
(B) REGISTRATION NUMBER: 33,215  
40 (C) REFERENCE/DOCKET NUMBER: NEX66/PCT
- (ix) TELECOMMUNICATION INFORMATION:
- (A) TELEPHONE: (303) 793-3333  
(B) TELEFAX: (303) 793-3433
- 45 (2) INFORMATION FOR SEQ ID NO: 1:
- (i) SEQUENCE CHARACTERIZATION:
- (A) LENGTH: 86 base pairs  
(B) TYPE: nucleic acid  
50 (C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (ii) MOLECULAR TYPE: DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:
- ATCCGCCTGA TTAGCGATAC TNNNNNNNNN NNNNNNNNNN NNNNNNNNNN 50  
NNNNNNNNNN NACTTGAGCA AAATCACCTG CAGGGG 86
- 55 (2) INFORMATION FOR SEQ ID NO: 2:
- (i) SEQUENCE CHARACTERIZATION:
- (A) LENGTH: 28 base pairs  
(B) TYPE: nucleic acid  
60 (C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (ii) MOLECULAR TYPE: DNA
- (ix) FEATURE:
- (D) OTHER INFORMATION: N at positions 1-3 is biotin
- 65 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:
- NNNCCCCTGC AGGTGATTTT GCTCAAGT 28

- (2) INFORMATION FOR SEQ ID NO: 3:  
(i) SEQUENCE CHARACTERIZATION:  
5 (A) LENGTH: 49 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: DNA  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:  
10 CCGAAGCTTA ATACGACTCA CTATAGGGAT CCGCCTGATT AGCGATACT 49
- (2) INFORMATION FOR SEQ ID NO: 4:  
(i) SEQUENCE CHARACTERIZATION:  
15 (A) LENGTH: 84 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: DNA  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:  
20 ATCCGCCTGA TTAGCGATAC TAGGCTTGAC AAAGGGCACC ATGGCTTAGT 50  
GGTCTAGTA CTTGAGCAAA ATCACCTGCA GGGG 84
- (2) INFORMATION FOR SEQ ID NO: 5:  
(i) SEQUENCE CHARACTERIZATION:  
25 (A) LENGTH: 85 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: DNA  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:  
30 ATCCGCCTGA TTAGCGATAC TCAGGGCACT GCAAGCAATT GTGGTCCCAA 50  
TGGGCTGAGT ACTTGAGCAA AATCACCTGC AGGGG 85
- (2) INFORMATION FOR SEQ ID NO: 6:  
(i) SEQUENCE CHARACTERIZATION:  
35 (A) LENGTH: 86 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: DNA  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:  
40 ATCCGCCTGA TTAGCGATAC TCCAGGCAGT CATGGTCATT GTTTACAGTC 50  
GTGGAGTAGG TACTTGAGCA AATCACCTGC CAGGGG 86
- (2) INFORMATION FOR SEQ ID NO: 7:  
(i) SEQUENCE CHARACTERIZATION:  
45 (A) LENGTH: 85 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: DNA  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:  
50 ATCCGCCTGA TTAGCGATAC TAGGTGATCC CTGCAAAGGC AGGATAACGT 50  
CCTGAGCATC ACTTGAGCAA AATCACCTGC AGGGG 85
- (2) INFORMATION FOR SEQ ID NO: 8:  
(i) SEQUENCE CHARACTERIZATION:  
60 (A) LENGTH: 83 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: DNA  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:  
65 ATCCGCCTGA TTAGCGATAC TATGTGATCC CTGCAGAGGG AGGANACGTC 50  
TGAGCATCAC TTGAGCAAAA TCACCTGCAG GGG 83

## 81

- (2) INFORMATION FOR SEQ ID NO: 9:  
(i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 86 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: DNA  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:  
ATCCGCCTGA TTAGCGATAC TCACGTGATC CCATAAGGGC TGCGAAAAT 50  
AGCAGAGCAT CACTTGAGCA AAATCACCTG CAGGGG 86
- (2) INFORMATION FOR SEQ ID NO: 10:  
(i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 86 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: DNA  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:  
ATCCGCCTGA TTAGCGATAC TGGTGACTA GAGGCGAGCA AACGATCCTT 50  
GGTTAGCGTC CACTTGAGCA AAATCACCTG CAGGGG 86
- (2) INFORMATION FOR SEQ ID NO: 11:  
(i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 85 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: DNA  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:  
ATCCGCCTGA TTAGCGATAC TGGTGCGACG AGGCTTACAC AAACGTACAC 50  
GTTTCCCCGC ACTTGAGCAA AATCACCTGC AGGGG 85
- (2) INFORMATION FOR SEQ ID NO: 12:  
(i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 86 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: DNA  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:  
ATCCGCCTGA TTAGCGATAC TTGTGCGAGC AGGGGCGTAC GAAAACCTTA 50  
CAGTTCCCCC GACTTGAGCA AAATCACCTG CAGGGG 86
- (2) INFORMATION FOR SEQ ID NO: 13:  
(i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 86 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: DNA  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:  
ATCCGCCTGA TTAGCGATAC TAGTGGAACA GGGCACGGAG AGTCAAACCTT 50  
TGGTTTCCCC CACTTGAGCA AAATCACCTG CAGGGG 86
- (2) INFORMATION FOR SEQ ID NO: 14:  
(i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 86 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: DNA  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:  
ATCCGCCTGA TTAGCGATAC TGTGGGTAGG GATCGGTGGA TGCCTCGTCA 50  
CTTCTAGTCC CACTTGAGCA AAATCACCTG CAGGGG 86

## 82

- (2) INFORMATION FOR SEQ ID NO: 15:  
(i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 86 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: DNA  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:  
ATCCGCCTGA TTAGCGATAC TGGGCGCCCT AAACAAAGGG TGGTCACTTC 50  
TAGTCCCAGG AACTTGAGCA AAATCACCTG CAGGGG 86
- (2) INFORMATION FOR SEQ ID NO: 16:  
(i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 87 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: DNA  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:  
ATCCGCCTGA TTAGCGATAC TTCCGGGCTC GGGATTCTGTG GTCACCTTCA 50  
GTCCCGGATA TAACTTGAGC AAAATCACCT GCAGGGG 87
- (2) INFORMATION FOR SEQ ID NO: 17:  
(i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 84 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: DNA  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:  
ATCCGCCTGA TTAGCGATAC TATGGGAGGG CGCGTTCTTC GTGGTTACTT 50  
TTAGTCCCGA CTTGAGCAAA ATCACCTGCA GGGG 84
- (2) INFORMATION FOR SEQ ID NO: 18:  
(i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 84 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: DNA  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:  
ATCCGCCTGA TTAGCGATAC TACGGGAGGG CACGTTCTTC GTGGTTACTT 50  
TTAGTCCCGA CTTGAGCAAA ATCACCTGCA GGGG 84
- (2) INFORMATION FOR SEQ ID NO: 19:  
(i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 86 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: DNA  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:  
ATCCGCCTGA TTAGCGATAC TGCTCGTAGG GGGCGATTCT TTCGCCGTTA 50  
CTCCAGTCC TACTTGAGCA AAATCACCTG CAGGGG 86
- (2) INFORMATION FOR SEQ ID NO: 20:  
(i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 86 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: DNA  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:  
ATCCGCCTGA TTAGCGATAC TGAGGCATGT TAACATGAGC ATCGTCTCAC 50  
GATCCTCAGC CACTTGAGCA AAATCACCTG CAGGGG 86

83

- (2) INFORMATION FOR SEQ ID NO: 21:
- (i) SEQUENCE CHARACTERIZATION:
- (A) LENGTH: 86 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ii) MOLECULAR TYPE: DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:
- ATCCGCCTGA TTAGCGATAC TCCACAGGCT ACGGCACGTA GAGCATCACC 50  
 ATGATCCTGT GACTTGAGCA AAATCACCTG CAGGGG 86
- (2) INFORMATION FOR SEQ ID NO: 22:
- (i) SEQUENCE CHARACTERIZATION:
- (A) LENGTH: 86 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ii) MOLECULAR TYPE: DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:
- ATCCGCCTGA TTAGCGATAC TGCGGGCATG GCACATGAGC ATCTCTGATC 50  
 CCGCAATCCT CACTTGAGCA AAATCACCTG CAGGGG 86
- (2) INFORMATION FOR SEQ ID NO: 23:
- (i) SEQUENCE CHARACTERIZATION:
- (A) LENGTH: 86 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ii) MOLECULAR TYPE: DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:
- ATCCGCCTGA TTAGCGATAC TACCGGGCTA CTTCGTAGAG CATCTCTGAT 50  
 CCCGGTGCTC GACTTGAGCA AAATCACCTG CAGGGG 86
- (2) INFORMATION FOR SEQ ID NO: 24:
- (i) SEQUENCE CHARACTERIZATION:
- (A) LENGTH: 86 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ii) MOLECULAR TYPE: DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:
- ATCCGCCTGA TTAGCGATAC TAAAGGGCGA ACGTAGGTCG AGGCATCCAT 50  
 TGGATCCCTT CACTTGAGCA AAATCACCTG CAGGGG 86
- (2) INFORMATION FOR SEQ ID NO: 25:
- (i) SEQUENCE CHARACTERIZATION:
- (A) LENGTH: 86 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ii) MOLECULAR TYPE: DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:
- ATCCGCCTGA TTAGCGATAC TACGGGCTCT GTCAGTGTGG CACTAGCAAT 50  
 AGTCCCGTCG CACTTGAGCA AAATCACCTG CAGGGG 86
- (2) INFORMATION FOR SEQ ID NO: 26:
- (i) SEQUENCE CHARACTERIZATION:
- (A) LENGTH: 85 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ii) MOLECULAR TYPE: DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:
- ATCCGCCTGA TTAGCGATAC TGGGCAGACC TTCTGGACGA GCATCACCTA 50  
 TGTGATCCCG ACTTGAGCAA AATCACCTGC AGGGG 85

## 84

- (2) INFORMATION FOR SEQ ID NO: 27:
- (i) SEQUENCE CHARACTERIZATION:
- (A) LENGTH: 86 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ii) MOLECULAR TYPE: DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:
- ATCCGCCTGA TTAGCGATAC TAGAGGGGAA GTAGGCTGCC TGA CTGAGAGA 50  
GAGTCCTCCC GACTTGAGCA AAATCACCTG CAGGGG 86
- (2) INFORMATION FOR SEQ ID NO: 28:
- (i) SEQUENCE CHARACTERIZATION:
- (A) LENGTH: 86 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ii) MOLECULAR TYPE: DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:
- ATCCGCCTGA TTAGCGATAC TAGGGGTGCG AAACACATAA TCCTCGCGGA 50  
TTCCCATCGC TACTTGAGCA AAATCACCTG CAGGGG 86
- (2) INFORMATION FOR SEQ ID NO: 29:
- (i) SEQUENCE CHARACTERIZATION:
- (A) LENGTH: 83 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ii) MOLECULAR TYPE: DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:
- ATCCGCCTGA TTAGCGATAC TGGGGGGGCA ATGGCGGTAC CTCTGGTCCC 50  
CTAAATACAC TTGAGCAAAA TCACCTGCAG GGG 83
- (2) INFORMATION FOR SEQ ID NO: 30:
- (i) SEQUENCE CHARACTERIZATION:
- (A) LENGTH: 85 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ii) MOLECULAR TYPE: DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:
- ATCCGCCTGA TTAGCGATAC TGCGGCTCAA AGTCCTGCTA CCCGAGCAC 50  
ATCTGTGGTC ACTTGAGCAA AATCACCTGC AGGGG 85
- (2) INFORMATION FOR SEQ ID NO: 31:
- (i) SEQUENCE CHARACTERIZATION:
- (A) LENGTH: 86 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ii) MOLECULAR TYPE: DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:
- ATCCGCCTGA TTAGCGATAC TTTGGGCGTG AATGTCCACG GGTACCTCCG 50  
GTCCCAAAGA GACTTGAGCA AAATCACCTG CAGGGG 86
- (2) INFORMATION FOR SEQ ID NO: 32:
- (i) SEQUENCE CHARACTERIZATION:
- (A) LENGTH: 85 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ii) MOLECULAR TYPE: DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:
- ATCCGCCTGA TTAGCGATAC TTCCGCGCAA GTCCTGGTA AAGGGCAGCC 50  
CTAACTGGTC ACTTGAGCAA AATCACCTGC AGGGG 85

## 85

- (2) INFORMATION FOR SEQ ID NO: 33:
- (i) SEQUENCE CHARACTERIZATION:
- (A) LENGTH: 86 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ii) MOLECULAR TYPE: DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:
- ATCCGCCTGA TTAGCGATAC TCAAGTCCC CACAAGACTG GGGCTGTTCA 50
- AACCGCTAGT AACTTGAGCA AAATCACCTG CAGGGG 86
- (2) INFORMATION FOR SEQ ID NO: 34:
- (i) SEQUENCE CHARACTERIZATION:
- (A) LENGTH: 86 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ii) MOLECULAR TYPE: DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:
- ATCCGCCTGA TTAGCGATAC TCAAGTAGGG CGCGACACAC GTCCGGGCAC 50
- CTAAGGTCCC AACTTGAGCA AAATCACCTG CAGGGG 86
- (2) INFORMATION FOR SEQ ID NO: 35:
- (i) SEQUENCE CHARACTERIZATION:
- (A) LENGTH: 86 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ii) MOLECULAR TYPE: DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35:
- ATCCGCCTGA TTAGCGATAC TAAAGTCGTG CAGGGTCCCC TGGAAGCATC 50
- TCCGATCCCA GACTTGAGCA AAATCACCTG CAGGGG 86
- (2) INFORMATION FOR SEQ ID NO: 36:
- (i) SEQUENCE CHARACTERIZATION:
- (A) LENGTH: 96 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ii) MOLECULAR TYPE: RNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 36:
- GGGAGACAAG AAUAACGCUC AANNNNNNNN NNNNNNNNNN NNNNNNNNNN 50
- NNNNNNNNNN NNNNNNNNNN NNUUCGACAG GAGGCUCACA ACAGGC 96
- (2) INFORMATION FOR SEQ ID NO: 37:
- (i) SEQUENCE CHARACTERIZATION:
- (A) LENGTH: 39 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ii) MOLECULAR TYPE: RNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 37:
- TAATACGACT CACTATAGGG AGACAAGAAT AACGCTCAA 39
- (2) INFORMATION FOR SEQ ID NO: 38:
- (i) SEQUENCE CHARACTERIZATION:
- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ii) MOLECULAR TYPE: RNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 38:
- GCCTGTTGTG AGCCTCCTGT CGAA 24
- (2) INFORMATION FOR SEQ ID NO: 39:
- (i) SEQUENCE CHARACTERIZATION:

## 86

- (A) LENGTH: 93 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear
- 5 (ii) MOLECULAR TYPE: RNA  
 (ix) FEATURE:  
 (D) OTHER INFORMATION: All pyrimidines are 2'-F modified  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 39:  
 GGGAGACAAG AAUAACGCUC AACGGUGGCA UUUCUUCACU UCCUUCUCGC 50  
 10 UUUCUCGCGU UGGGCNCGAU UCGACAGGAG GCUCACAACA GGC 93
- (2) INFORMATION FOR SEQ ID NO: 40:  
 (i) SEQUENCE CHARACTERIZATION:  
 15 (A) LENGTH: 91 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear  
 (ii) MOLECULAR TYPE: RNA  
 (ix) FEATURE:  
 20 (D) OTHER INFORMATION: All pyrimidines are 2'-F modified  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 40:  
 GGGAGACAAG AAUAACGCUC AACCAACCUU CUGUCGGCGU UGCUUUUUGG 50  
 ACGGCACUCA GGCUCCAUUC GACAGGAGGC UCACAACAGG C 91
- 25 (2) INFORMATION FOR SEQ ID NO: 41:  
 (i) SEQUENCE CHARACTERIZATION:  
 (A) LENGTH: 95 base pairs  
 (B) TYPE: nucleic acid  
 30 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear  
 (ii) MOLECULAR TYPE: RNA  
 (ix) FEATURE:  
 (D) OTHER INFORMATION: All pyrimidines are 2'-F modified  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 41:  
 35 GGGAGACAAG AAUAACGCUC AAUCGAUCGG UUGUGUGCCG GACAGCCUUA 50  
 ACCAGGGCUG GGACCGAGGC CUUCGACAGG AGGCUCACAA CAGGC 95
- (2) INFORMATION FOR SEQ ID NO: 42:  
 (i) SEQUENCE CHARACTERIZATION:  
 40 (A) LENGTH: 92 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear  
 (ii) MOLECULAR TYPE: RNA  
 45 (ix) FEATURE:  
 (D) OTHER INFORMATION: All pyrimidines are 2'-F modified  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 42:  
 GGGAGACAAG AAUAACGCUC AACUGAGUAG GGGAGGAAGU UGAAUCAGUU 50  
 GUGGCGCCUC UCAUUCGCUU CGACAGGAGG CUCACAACAG GC 92
- 50 (2) INFORMATION FOR SEQ ID NO: 43:  
 (i) SEQUENCE CHARACTERIZATION:  
 (A) LENGTH: 95 base pairs  
 55 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear  
 (ii) MOLECULAR TYPE: RNA  
 (ix) FEATURE:  
 (D) OTHER INFORMATION: All pyrimidines are 2'-F modified  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 43:  
 60 GGGAGACAAG AAUAACGCUC AACAGCACUU UCGCUUUUCA UCAUUUUUUC 50  
 UUUCCACUGU UGGGCGCGGA AUUCGACAGG AGGCUCACAA CAGGC 95
- 65 (2) INFORMATION FOR SEQ ID NO: 44:  
 (i) SEQUENCE CHARACTERIZATION:  
 (A) LENGTH: 96 base pairs



87

- (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear
- (ii) MOLECULAR TYPE: RNA
- (ix) FEATURE:  
 (D) OTHER INFORMATION: All pyrimidines are 2'-F modified
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 44:  
 GGGAGACAAG AAUAACGCUC AAUCAGUGCU GGCGUCAUGU CUCGAUGGGG 50  
 AUUUUUUCUUC AGCACUUUGC CAUUCGACAG GAGGCUCACA ACAGGC 96
- (2) INFORMATION FOR SEQ ID NO: 45:  
 (i) SEQUENCE CHARACTERIZATION:  
 (A) LENGTH: 96 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear
- (ii) MOLECULAR TYPE: RNA
- (ix) FEATURE:  
 (D) OTHER INFORMATION: All pyrimidines are 2'-F modified
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 45:  
 GGGAGACAAG AAUAACGCUC AAUCUACUUU CCAUUUCUCU UUUCUUCUCA 50  
 CGAGCGGGUU UCCAGUGAAC CAUUCGACAG GAGGCUCACA ACAGGC 96
- (2) INFORMATION FOR SEQ ID NO: 46:  
 (i) SEQUENCE CHARACTERIZATION:  
 (A) LENGTH: 94 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear
- (ii) MOLECULAR TYPE: RNA
- (ix) FEATURE:  
 (D) OTHER INFORMATION: All pyrimidines are 2'-F modified
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 46:  
 GGGAGACAAG AAUAACGCUC AACGAUAGUG ACUACGAUGA CGAAGGCCGC 50  
 GGGUUGGAUG CCCGCAUUGA UUCGACAGGA GGCUCACAAC AGGC 94
- (2) INFORMATION FOR SEQ ID NO: 47:  
 (i) SEQUENCE CHARACTERIZATION:  
 (A) LENGTH: 93 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear
- (ii) MOLECULAR TYPE: RNA
- (ix) FEATURE:  
 (D) OTHER INFORMATION: All pyrimidines are 2'-F modified
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 47:  
 GGGAGACAAG AAUAACGCUC AAGUCGAUAC UGGCGACUUG CUCCAUUGGC 50  
 CGAUUAACGA UUCGGUCAGU UCGACAGGAG GCUCACAACA GGC 93
- (2) INFORMATION FOR SEQ ID NO: 48:  
 (i) SEQUENCE CHARACTERIZATION:  
 (A) LENGTH: 95 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear
- (ii) MOLECULAR TYPE: RNA
- (ix) FEATURE:  
 (D) OTHER INFORMATION: All pyrimidines are 2'-F modified
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 48:  
 GGGAGACAAG AAUAACGCUC AAGUGCAAAC UUAACCCGGG AACC GCGCGU 50  
 UUCGAUCGAC UUUCUUUCC AUUCGACAGG AGGCUCACAA CAGGC 95
- (2) INFORMATION FOR SEQ ID NO: 49:  
 (i) SEQUENCE CHARACTERIZATION:  
 (A) LENGTH: 96 base pairs  
 (B) TYPE: nucleic acid

88

- (C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: RNA  
(ix) FEATURE:  
(D) OTHER INFORMATION: All pyrimidines are 2'-F modified  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 49:  
GGGAGACAAG AAUAACGCUC AAUUCGCG UUCGGAUUA UCCUGUGCUC 50  
GGAAAUCGGU AGCCAUAGUG CAUUCGACAG GAGGCUCACA ACAGGC 96
- (2) INFORMATION FOR SEQ ID NO: 50:  
(i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 94 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: RNA  
(ix) FEATURE:  
(D) OTHER INFORMATION: All pyrimidines are 2'-F modified  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 50:  
GGGAGACAAG AAUAACGCUC AACGAACGAG GAGGGAGUGG CAAGGGAUGG 50  
UUGGAUAGGC UCUACGCUCA UUCGACAGGA GGCUCACAAC AGGC 94
- (2) INFORMATION FOR SEQ ID NO: 51:  
(i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 94 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: RNA  
(ix) FEATURE:  
(D) OTHER INFORMATION: All pyrimidines are 2'-F modified  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 51:  
GGGAGACAAG AAUAACGCUC AAGCGAAACU GGCACUUGC UCCAUUGGCC 50  
GAUAUAACGA UUCGGUUCAU UUCGACAGGA GGCUCACAAC AGGC 94
- (2) INFORMATION FOR SEQ ID NO: 52:  
(i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 95 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: RNA  
(ix) FEATURE:  
(D) OTHER INFORMATION: All pyrimidines are 2'-F modified  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 52:  
GGGAGACAAG AAUAACGCUC AACGAACGAG GAGGGAGUCG CAAGGGAUGG 50  
UUGGAUAGGC UCUACGCUCA AUUCGACAGG AGGCUCACAA CAGGC 95
- (2) INFORMATION FOR SEQ ID NO: 53:  
(i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 94 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: RNA  
(ix) FEATURE:  
(D) OTHER INFORMATION: All pyrimidines are 2'-F modified  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 53:  
GGGAGACAAG AAUAACGCUC AACGAGAAGU GACUACGAUG ACGAAGGCCG 50  
CGGGUUGAAU CCCUCAUUGA UUCGACAGGA GGCUCACAAC AGGC 94
- (2) INFORMATION FOR SEQ ID NO: 54:  
(i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 95 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single

- (D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: RNA  
(ix) FEATURE:  
(D) OTHER INFORMATION: All pyrimidines are 2'-F modified
- 5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 54:  
GGGAGACAAG AAUAACGCUC AAAAGCAACG AGACCUGACG CCUGAUGUGA 50  
CUGUGCUUGC ACCCGAUUCU GUUCGACAGG AGGCUCACAA CAGGC 95
- (2) INFORMATION FOR SEQ ID NO: 55:  
10 (i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 96 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
15 (ii) MOLECULAR TYPE: RNA  
(ix) FEATURE:  
(D) OTHER INFORMATION: All pyrimidines are 2'-F modified  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 55:  
GGGAGACAAG AAUAACGCUC AAGUGAUUCU CAUUCUCAAU GCUUUCUCAC 50  
20 AACUUUUUCC ACUUCAGCGU GAUUCGACAG GAGGCUCACA CAGGC 96
- (2) INFORMATION FOR SEQ ID NO: 56:  
(i) SEQUENCE CHARACTERIZATION:  
25 (A) LENGTH: 94 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: RNA  
30 (ix) FEATURE:  
(D) OTHER INFORMATION: All pyrimidines are 2'-F modified  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 56:  
GGGAGACAAG AAUAACGCUC AAAAGCAACG AGACUCGACG CCUGAUGUGA 50  
CUGUGCUUGC ACCCGAUUCU UUCGACAGGA GGCUCACAAC AGGC 94
- 35 (2) INFORMATION FOR SEQ ID NO: 57:  
(i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 96 base pairs  
(B) TYPE: nucleic acid  
40 (C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: RNA  
(ix) FEATURE:  
(D) OTHER INFORMATION: All pyrimidines are 2'-F modified  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 57:  
45 GGGAGACAAG AAUAACGCUC AAUCGAUCGG UUGUGGCCG GACAGCUUUG 50  
ACCAUGAGCU GGGACCGAGG CCUUCGACAG GAGGCUCACA ACAGGC 96
- (2) INFORMATION FOR SEQ ID NO: 58:  
50 (i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 96 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: RNA  
55 (ix) FEATURE:  
(D) OTHER INFORMATION: All pyrimidines are 2'-F modified  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 58:  
GGGAGACAAG AAUAACGCUC AANGACNGU GGACCUGACU AAUCGACUGA 50  
UCAAGAUAUC CGCCCAUG GGUUCGACAG GAGGCUCACA ACAGGC 96
- 60 (2) INFORMATION FOR SEQ ID NO: 59:  
(i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 94 base pairs  
65 (B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

- (ii) MOLECULAR TYPE: RNA  
(ix) FEATURE:  
(D) OTHER INFORMATION: All pyrimidines are 2'-F modified  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 59:  
5 GGGAGACAAG AAUAACGCUC AACACUGCGA CUUGCAGAAG CCUUGUGUGG 50  
CGGUACCCCC UUUGGCCUCG UUCGACAGGA GGCUCACAAC AGGC 94
- (2) INFORMATION FOR SEQ ID NO: 60:  
(i) SEQUENCE CHARACTERIZATION:  
10 (A) LENGTH: 94 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: RNA  
15 (ix) FEATURE:  
(D) OTHER INFORMATION: All pyrimidines are 2'-F modified  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 60:  
GGGAGACAAG AAUAACGCUC AAGGUGGCAU UUCUUCAUUU UCCUUCUCGC 50  
UUUCUCCGCC GUUGGGCGCG UUCGACAGGA GGCUCACAAC AGGC 94
- (2) INFORMATION FOR SEQ ID NO: 61:  
(i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 96 base pairs  
(B) TYPE: nucleic acid  
25 (C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: RNA  
(ix) FEATURE:  
(D) OTHER INFORMATION: All pyrimidines are 2'-F modified  
30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 61:  
GGGAGACAAG AAUAACGCUC AACCUGAGUA GGGGGGAAAG UUGAAUCAGU 50  
UGUGGCGCUC UACUCAUUCG CCUUCGACAG GAGGCUCACA ACAGGC 96
- (2) INFORMATION FOR SEQ ID NO: 62:  
35 (i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 94 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
40 (ii) MOLECULAR TYPE: RNA  
(ix) FEATURE:  
(D) OTHER INFORMATION: All pyrimidines are 2'-F modified  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 62:  
GGGAGACAAG AAUAACGCUC AAGUCGAAAC UGGCGACUUG CUCCAUUGGC 50  
45 CGAUUAACG AUUCGGUUA UUCGACAGGA GGCUCACAAC AGGC 94
- (2) INFORMATION FOR SEQ ID NO: 63:  
(i) SEQUENCE CHARACTERIZATION:  
50 (A) LENGTH: 94 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: RNA  
(ix) FEATURE:  
55 (D) OTHER INFORMATION: All pyrimidines are 2'-F modified  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 63:  
GGGAGACAAG AAUAACGCUC AAGCGAUACU GGCGACUUGC CUCCAUUGGCC 50  
GAUAUAACGA UUCGGCUCAG UUCGACAGGA GGCUCACAAC AGGC 94
- (2) INFORMATION FOR SEQ ID NO: 64:  
(i) SEQUENCE CHARACTERIZATION:  
60 (A) LENGTH: 96 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
65 (D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: RNA

- (ix) FEATURE:  
 (D) OTHER INFORMATION: All pyrimidines are 2'-F modified
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 64:  
 5 GGGAGACAAG AAUAACGCUC AAACGUGGGG CACAGGACCG AGAGUCCCUC 50  
 CGGCAUAGC CGCUACCCCA CCUUCGACAG GAGGCUCACA ACAGGC 96
- (2) INFORMATION FOR SEQ ID NO: 65:  
 (i) SEQUENCE CHARACTERIZATION:  
 10 (A) LENGTH: 98 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear  
 (ii) MOLECULAR TYPE: RNA  
 (ix) FEATURE:  
 15 (D) OTHER INFORMATION: All pyrimidines are 2'-F modified  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 65:  
 GGGAGACAAG AAUAACGCUC AACACAGCCU NANAGGGGGG AAGUUGAAUC 50  
 AGUUGUGGCG CUCUACUCAU UCGCUUCGAC AGGAGGCUCA CAACAGGC 98
- (2) INFORMATION FOR SEQ ID NO: 66:  
 (i) SEQUENCE CHARACTERIZATION:  
 20 (A) LENGTH: 94 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 25 (D) TOPOLOGY: linear  
 (ii) MOLECULAR TYPE: RNA  
 (ix) FEATURE:  
 (D) OTHER INFORMATION: All pyrimidines are 2'-F modified  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 66:  
 30 GGGAGACAAG AAUAACGCUC AAANGGGNUA UGGUGACUUG CUCCAUUGGC 50  
 CGAUUAUACG AUUCGGUCAG UUCGACAGGA GGCUCACAAC AGGC 94
- (2) INFORMATION FOR SEQ ID NO: 67:  
 35 (i) SEQUENCE CHARACTERIZATION:  
 (A) LENGTH: 96 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear  
 (ii) MOLECULAR TYPE: RNA  
 40 (ix) FEATURE:  
 (D) OTHER INFORMATION: All pyrimidines are 2'-F modified  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 67:  
 GGGAGACAAG AAUAACGCUC AACUGCGUA GGGNGGGAAG UUGAAUCAGU 50  
 45 UGUGGCGCUC UACUCAUUCG CCUUCGACAG GAGGCUCACA ACAGGC 96
- (2) INFORMATION FOR SEQ ID NO: 68:  
 (i) SEQUENCE CHARACTERIZATION:  
 50 (A) LENGTH: 94 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear  
 (ii) MOLECULAR TYPE: RNA  
 (ix) FEATURE:  
 55 (D) OTHER INFORMATION: All pyrimidines are 2'-F modified  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 68:  
 GGGAGACAAG AAUAACGCUC AACGAACGAG GAGGGAGUGG CAAGGGAUGG 50  
 UUGGAUAGGC UCUACGCUCA UUCGACAGGA GGCUCACAAC AGGC 94
- (2) INFORMATION FOR SEQ ID NO: 69:  
 60 (i) SEQUENCE CHARACTERIZATION:  
 (A) LENGTH: 97 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear  
 65 (ii) MOLECULAR TYPE: RNA  
 (ix) FEATURE:

## 92

- (D) OTHER INFORMATION: All pyrimidines are 2'-F modified
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 69:  
 GGGAGACAAG AAUAACGCUC AAGUGCAAAC UUAACCCGGG AACCGCGCGU 50  
 UUCGAUUCGC UUUCNUAAU CCAUUCGACA GGAGGCUCAC AACAGGC 97
- 5 (2) INFORMATION FOR SEQ ID NO: 70:
- (i) SEQUENCE CHARACTERIZATION:  
 (A) LENGTH: 93 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear
- 10 (ii) MOLECULAR TYPE: RNA
- (ix) FEATURE:  
 (D) OTHER INFORMATION: All pyrimidines are 2'-F modified
- 15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 70:  
 GGGAGACAAG AAUAACGCUC AACGAACGAG GAGGAGUGG CAAGGGACGG 50  
 UNNAUAGGCU CUACGCUCAU UCGACAGGAG GCUCACAACA GGC 93
- (2) INFORMATION FOR SEQ ID NO: 71:
- 20 (i) SEQUENCE CHARACTERIZATION:  
 (A) LENGTH: 93 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear
- 25 (ii) MOLECULAR TYPE: RNA
- (ix) FEATURE:  
 (D) OTHER INFORMATION: All pyrimidines are 2'-F modified
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 71:  
 GGGAGACAAG AAUAACGCUC AAUCGGUGUG GCUCAGAAAC UGACACGCGU 50  
 30 GAGCUUCGCA CACAUCUGCU UCGACAGGAG GCUCACAACA GGC 93
- (2) INFORMATION FOR SEQ ID NO: 72:
- (i) SEQUENCE CHARACTERIZATION:  
 (A) LENGTH: 95 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear
- 35 (ii) MOLECULAR TYPE: RNA
- (ix) FEATURE:  
 (D) OTHER INFORMATION: All pyrimidines are 2'-F modified
- 40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 72:  
 GGGAGACAAG AAUAACGCUC AAUAUCGCUU UUCAUCAAU CCACUUUUUC 50  
 ACUCUNUAAC UUGGGCGUGC AUUCGACAGG AGGCUCACAA CAGGC 95
- 45 (2) INFORMATION FOR SEQ ID NO: 73:
- (i) SEQUENCE CHARACTERIZATION:  
 (A) LENGTH: 96 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear
- 50 (ii) MOLECULAR TYPE: RNA
- (ix) FEATURE:  
 (D) OTHER INFORMATION: All pyrimidines are 2'-F modified
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 73:  
 55 GGGAGACAAG AAUAACGCUC AAGUGCAAAC UUAACCCGGG AACCGCGCGU 50  
 UUCGAUCCUG CAUCCUUUUU CCUUCGACAG GAGGCUCACA ACAGGC 96
- (2) INFORMATION FOR SEQ ID NO: 74:
- 60 (i) SEQUENCE CHARACTERIZATION:  
 (A) LENGTH: 93 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear
- (ii) MOLECULAR TYPE: RNA
- 65 (ix) FEATURE:  
 (D) OTHER INFORMATION: All pyrimidines are 2'-F modified

## 93

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 74:  
 GGGAGACAAG AAUAACGCUC AAUCGUCGG UUGUGUGCCG GCAGCUUUGU  
 CCAGCGUUGG GCCGAGGCCU UCGACAGGAG GCUCACAACA GGC

93

50

## 5 (2) INFORMATION FOR SEQ ID NO: 75:

## (i) SEQUENCE CHARACTERIZATION:

- (A) LENGTH: 95 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

10

## (ii) MOLECULAR TYPE: RNA

## (ix) FEATURE:

- (D) OTHER INFORMATION: All pyrimidines are 2'-F modified

## 15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 75:

GGGAGACAAG AAUAACGCUC AAAGUACCCA UCUCAUUUU UCCUUUCCUU  
 UCUUCAAGGC ACAUUGAGGG UUUCGACAGG AGGUCACAA CAGGC

50

95

## (2) INFORMATION FOR SEQ ID NO: 76:

## (i) SEQUENCE CHARACTERIZATION:

- (A) LENGTH: 97 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

20

## (ii) MOLECULAR TYPE: RNA

## (ix) FEATURE:

- (D) OTHER INFORMATION: All pyrimidines are 2'-F modified

25

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 76:

GGGAGACAAG AAUAACGCUC AACCGAGUA GGGGGGAAG UUGAACCAGU  
 UGUGGCNGCC UACUCAUUCN CCAUUCGACA GGAGGCUCAC AACAGGC

50

97

30

## (2) INFORMATION FOR SEQ ID NO: 77:

## (i) SEQUENCE CHARACTERIZATION:

- (A) LENGTH: 94 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

35

## (ii) MOLECULAR TYPE: RNA

## (ix) FEATURE:

- (D) OTHER INFORMATION: All pyrimidines are 2'-F modified

40

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 77:

GGGAGACAAG AAUAACGCUC AACCNCCUN CUGUCGGCGC UUGUCUUUUU  
 GGACGGGCAA CCCAGGGCUC UUCGACAGGA GGCUCACAAC AGGC

50

94

## 45 (2) INFORMATION FOR SEQ ID NO: 78:

## (i) SEQUENCE CHARACTERIZATION:

- (A) LENGTH: 96 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

50

## (ii) MOLECULAR TYPE: RNA

## (ix) FEATURE:

- (D) OTHER INFORMATION: All pyrimidines are 2'-F modified

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 78:

GGGAGACAAG AAUAACGCUC AACCAACCUN CUGUCGGCGC UUGUCUUUUU  
 GGACGAGCAA CUCAAGGCUC GUUUCGACAG GAGGCUCACA ACAGGC

50

96

55

## (2) INFORMATION FOR SEQ ID NO: 79:

## (i) SEQUENCE CHARACTERIZATION:

- (A) LENGTH: 93 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

60

## (ii) MOLECULAR TYPE: RNA

## (ix) FEATURE:

- (D) OTHER INFORMATION: All pyrimidines are 2'-F modified

65

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 79:

GGGAGACAAG AAUAACGCUC AACCAGCGCA GAUCCCGGGC UGAAGUGACU 50  
 GCCGGCAACG GCCGCUCCAU UCGACAGGAG GCUCACAACA GGC 93

- (2) INFORMATION FOR SEQ ID NO: 80:
- 5 (i) SEQUENCE CHARACTERIZATION:  
 (A) LENGTH: 96 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear
- 10 (ii) MOLECULAR TYPE: RNA  
 (ix) FEATURE:  
 (D) OTHER INFORMATION: All pyrimidines are 2'-F modified
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 80:  
 GGGAGACAAG AAUAACGCUC AAUCCCGUA ACAACUUUUC AUUUUCACUU 50  
 15 UUCAUCCAAC CAGUGAGCAG CAUUCGACAG GAGGCUCACA ACAGGC 96
- (2) INFORMATION FOR SEQ ID NO: 81:
- 20 (i) SEQUENCE CHARACTERIZATION:  
 (A) LENGTH: 96 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear
- (ii) MOLECULAR TYPE: RNA  
 (ix) FEATURE:  
 (D) OTHER INFORMATION: All pyrimidines are 2'-F modified
- 25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 81:  
 GGGAGACAAG AAUAACGCUC AAUAUCGCUU UCAUCAAAUU CCACUCCUUC 50  
 ACUUCUUUAA CUUGGGCGUG CAUUCGACAG GAGGCUCACA ACAGGC 96
- 30 (2) INFORMATION FOR SEQ ID NO: 82:
- (i) SEQUENCE CHARACTERIZATION:  
 (A) LENGTH: 23 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 35 (D) TOPOLOGY: linear
- (ii) MOLECULAR TYPE: DNA  
 (ix) FEATURE:  
 (D) OTHER INFORMATION: N at positions 1 and 23 is any base  
 pair.
- 40 (ix) FEATURE:  
 (D) OTHER INFORMATION: N at positions 5 and 10 is any base  
 pair.
- (ix) FEATURE:  
 (D) OTHER INFORMATION: N at positions 6 and 9 is any base  
 45 pair.
- (ix) FEATURE:  
 (D) OTHER INFORMATION: N at positions 7 and 8 is any base  
 pair.
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 82:  
 50 NGGCNNNNNN GRKYAYRRT CCN 23
- (2) INFORMATION FOR SEQ ID NO: 83:
- (i) SEQUENCE CHARACTERIZATION:  
 (A) LENGTH: 38 base pairs  
 55 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear
- (ii) MOLECULAR TYPE: DNA  
 (ix) FEATURE:  
 (D) OTHER INFORMATION: Nucleotide 38 is an inverted  
 60 orientation T (3'-3'-linked)
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 83:  
 TGGGAGGGCG CGTTCTTCGT GGTACTTTT AGTCCCGT 38
- 65 (2) INFORMATION FOR SEQ ID NO: 84:
- (i) SEQUENCE CHARACTERIZATION:



95

- (A) LENGTH: 40 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- 5 (ii) MOLECULAR TYPE: DNA  
(ix) FEATURE:  
(D) OTHER INFORMATION: Nucleotide 40 is an inverted orientation T (3'-3'-linked)
- 10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 84:  
CACAGGCTAC GGCACGTAGA GCATCACCAT GATCCTGTGT 40
- (2) INFORMATION FOR SEQ ID NO: 85:  
(i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 45 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- 15 (ii) MOLECULAR TYPE: DNA  
(ix) FEATURE:  
(D) OTHER INFORMATION: Nucleotide 45 is an inverted orientation T (3'-3'-linked)
- 20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 85:  
TACTCAGGGC ACTGCAAGCA ATTGTGGTCC CAATGGGCTC AGTAT 45
- 25 (2) INFORMATION FOR SEQ ID NO: 86:  
(i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 36 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- 30 (ii) MOLECULAR TYPE: DNA  
(ix) FEATURE:  
(D) OTHER INFORMATION: C at positions 11, 25 and 26 is 2'-O-Methyl-2'-deoxycytidine.
- 35 (ix) FEATURE:  
(D) OTHER INFORMATION: G at positions 9, 10, 17, 19 and 35 is 2'-O-Methyl-2'-deoxyguanosine.
- 40 (ix) FEATURE:  
(D) OTHER INFORMATION: A at positions 12, 24 and 27 is 2'-O-Methyl-2'-deoxyadenosine.
- 45 (ix) FEATURE:  
(D) OTHER INFORMATION: U at positions 6, 22 and 34 is 2'-fluoro-2'-deoxyuridine.
- 50 (ix) FEATURE:  
(D) OTHER INFORMATION: C at positions 8, 23, 32 and 33 is 2'-fluoro-2'-deoxycytidine.
- 50 (ix) FEATURE:  
(D) OTHER INFORMATION: Nucleotide 36 is an inverted orientation T (3'-3'-linked).
- 50 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 86:  
CAGGCUACGG CACGTAGAGC AUCACCATGA TCCUGT 36
- (2) INFORMATION FOR SEQ ID NO: 87:  
(i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 32 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- 55 (ii) MOLECULAR TYPE: DNA  
(ix) FEATURE:  
(D) OTHER INFORMATION: G at positions 9, 15, 17 and 31 is 2'-O-methyl-2'-deoxyguanosine.
- 60 (ix) FEATURE:  
(D) OTHER INFORMATION: A at position 22 is 2'-O-methyl-2'-deoxyadenine.
- 65 (ix) FEATURE:

## 96

- (D) OTHER INFORMATION: U at positions 6, 20 and 30 is 2'-fluoro-2'-deoxyuridine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: C at positions 8, 21, 28 and 29 is 2'-fluoro-2'-deoxycytidine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: N at positions 10 and 23 is hexaethylene glycol phosphoramidite spacer.  
(ix) FEATURE:  
(D) OTHER INFORMATION: Nucleotide 32 is an inverted orientation T (3'-3'-linked).  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 87:  
CAGGCUACGN CGTAGAGCAU CANTGATCCU GT 32
- (2) INFORMATION FOR SEQ ID NO: 88:  
(i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 39 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: DNA  
(ix) FEATURE:  
(D) OTHER INFORMATION: Nucleotide 39 is an inverted orientation T (3'-3'-linked).  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 88:  
CAGTCCGTGG TAGGGCAGGT TGGGGTGACT TCGTGAAT 39
- (2) INFORMATION FOR SEQ ID NO: 89:  
(i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 37 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: DNA  
(ix) FEATURE:  
(D) OTHER INFORMATION: T at positions 13, 14, 16 and 17 is substituted with IdU.  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 89:  
TGGGAGGGCG CGTTCTTCGT GGTACTTTT AGTCCCG 37
- (2) INFORMATION FOR SEQ ID NO: 90:  
(i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 37 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: DNA  
(ix) FEATURE:  
(D) OTHER INFORMATION: T at position 20 is substituted with IdU.  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 90:  
TGGGAGGGCG CGTTCTTCGT GGTACTTTT AGTCCCG 37
- (2) INFORMATION FOR SEQ ID NO: 91:  
(i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 37 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: DNA  
(ix) FEATURE:  
(D) OTHER INFORMATION: T at position 23 is substituted with IdU.  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 91:  
TGGGAGGGCG CGTTCTTCGT GGTACTTTT AGTCCCG 37

## 97

- (2) INFORMATION FOR SEQ ID NO: 92:  
 (i) SEQUENCE CHARACTERIZATION:  
 (A) LENGTH: 37 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear  
 (ii) MOLECULAR TYPE: DNA  
 (ix) FEATURE:  
 (D) OTHER INFORMATION: T at position 24 is substituted with  
 IdU.  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 92:  
 TGGGAGGGCG CGTTCTTCGT GGTACTTTT AGTCCCG 37
- (2) INFORMATION FOR SEQ ID NO: 93:  
 (i) SEQUENCE CHARACTERIZATION:  
 (A) LENGTH: 37 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear  
 (ii) MOLECULAR TYPE: DNA  
 (ix) FEATURE:  
 (D) OTHER INFORMATION: T at position 27 is substituted with  
 IdU.  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 93:  
 TGGGAGGGCG CGTTCTTCGT GGTACTTTT AGTCCCG 37
- (2) INFORMATION FOR SEQ ID NO: 94:  
 (i) SEQUENCE CHARACTERIZATION:  
 (A) LENGTH: 37 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear  
 (ii) MOLECULAR TYPE: DNA  
 (ix) FEATURE:  
 (D) OTHER INFORMATION: T at positions 28-30 is substituted with  
 IdU.  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 94:  
 TGGGAGGGCG CGTTCTTCGT GGTACTTTT AGTCCCG 37
- (2) INFORMATION FOR SEQ ID NO: 95:  
 (i) SEQUENCE CHARACTERIZATION:  
 (A) LENGTH: 37 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear  
 (ii) MOLECULAR TYPE: DNA  
 (ix) FEATURE:  
 (D) OTHER INFORMATION: T at position 33 is substituted with  
 IdU.  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 95:  
 TGGGAGGGCG CGTTCTTCGT GGTACTTTT AGTCCCG 37
- (2) INFORMATION FOR SEQ ID NO: 96:  
 (i) SEQUENCE CHARACTERIZATION:  
 (A) LENGTH: 7 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear  
 (ii) MOLECULAR TYPE: Peptide  
 (ix) FEATURE:  
 (D) OTHER INFORMATION: Xaa at position 5 is a modified  
 amino acid that could not be identified.  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 96:  
 Lys Lys Pro Ile Xaa Lys Lys

## 98

- (2) INFORMATION FOR SEQ ID NO: 97:
- (i) SEQUENCE CHARACTERIZATION:
- (A) LENGTH: 40 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ii) MOLECULAR TYPE: DNA
- (ix) FEATURE:
- (D) OTHER INFORMATION: C at positions 1 and 3 is 2'-O-Methyl-2'-deoxycytidine.
- (ix) FEATURE:
- (D) OTHER INFORMATION: A at position 2 is 2'-O-Methyl-2'-deoxyadenosine.
- (ix) FEATURE:
- (D) OTHER INFORMATION: G at positions 37 and 39 is 2'-O-Methyl-2'-deoxyguanosine.
- (ix) FEATURE:
- (D) OTHER INFORMATION: U at position 38 is 2'-O-Methyl-2'-deoxyuridine.
- (ix) FEATURE:
- (D) OTHER INFORMATION: Nucleotide 40 is an inverted orientation T (3'-3'-linked).
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 97:
- CACAGGCTAC GGCACGTAGA GCATCACCAT GATCCTGUGT 40
- (2) INFORMATION FOR SEQ ID NO: 98:
- (i) SEQUENCE CHARACTERIZATION:
- (A) LENGTH: 40 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ii) MOLECULAR TYPE: DNA
- (ix) FEATURE:
- (D) OTHER INFORMATION: C at positions 10, 13 and 15 is 2'-O-Methyl-2'-deoxycytidine.
- (ix) FEATURE:
- (D) OTHER INFORMATION: G at positions 11, 12 and 16 is 2'-O-Methyl-2'-deoxyguanosine.
- (ix) FEATURE:
- (D) OTHER INFORMATION: A at position 14 is 2'-O-Methyl-2'-deoxyadenosine.
- (ix) FEATURE:
- (D) OTHER INFORMATION: U at position 17 is 2'-O-Methyl-2'-deoxyuridine.
- (ix) FEATURE:
- (D) OTHER INFORMATION: Nucleotide 40 is an inverted orientation T (3'-3'-linked).
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 98:
- CACAGGCTAC GGCACGUAGA GCATCACCAT GATCCTGTGT 40
- (2) INFORMATION FOR SEQ ID NO: 99:
- (i) SEQUENCE CHARACTERIZATION:
- (A) LENGTH: 40 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ii) MOLECULAR TYPE: DNA
- (ix) FEATURE:
- (D) OTHER INFORMATION: A at positions 26 and 29 is 2'-O-Methyl-2'-deoxyadenosine.
- (ix) FEATURE:
- (D) OTHER INFORMATION: C at positions 27 and 28 is 2'-O-Methyl-2'-deoxycytidine.
- (ix) FEATURE:
- (D) OTHER INFORMATION: U at position 30 is 2'-O-Methyl-2'-deoxyuridine.

- (ix) FEATURE:  
(D) OTHER INFORMATION: Nucleotide 40 is an inverted orientation T (3'-3'-linked).
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 99:  
5 CACAGGCTAC GGCACGTAGA GCATCACCAU GATCCTGTGT 40
- (2) INFORMATION FOR SEQ ID NO: 100:  
(i) SEQUENCE CHARACTERIZATION:  
10 (A) LENGTH: 40 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: DNA  
(ix) FEATURE:  
15 (D) OTHER INFORMATION: C at positions 1, 3, 10, 13, 15, 27, and 28 is 2'-O-Methyl-2'-deoxycytidine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: A at positions 2, 14, 26 and 29 is 2'-O-Methyl-2'-deoxyadenosine.  
20 (ix) FEATURE:  
(D) OTHER INFORMATION: G at positions 11, 12 and 16 is 2'-O-Methyl-2'-deoxyguanosine.  
(ix) FEATURE:  
25 (D) OTHER INFORMATION: U at positions 17 and 30 is 2'-O-Methyl-2'-deoxyuridine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: Nucleotide 40 is an inverted orientation T (3'-3'-linked).
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 100:  
30 CACAGGCTAC GGCACGUAGA GCATCACCAU GATCCTGTGT 40
- (2) INFORMATION FOR SEQ ID NO: 101:  
(i) SEQUENCE CHARACTERIZATION:  
35 (A) LENGTH: 40 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: DNA  
(ix) FEATURE:  
40 (D) OTHER INFORMATION: C at positions 25, 27 and 28 is 2'-O-Methyl-2'-deoxycytidine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: A at positions 26 and 29 is 2'-O-Methyl-2'-deoxyadenosine.  
45 (ix) FEATURE:  
(D) OTHER INFORMATION: Nucleotide 40 is an inverted orientation T (3'-3'-linked).
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 101:  
50 CACAGGCTAC GGCACGTAGA GCATCACCAT GATCCTGTGT 40
- (2) INFORMATION FOR SEQ ID NO: 102:  
(i) SEQUENCE CHARACTERIZATION:  
55 (A) LENGTH: 40 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: DNA  
(ix) FEATURE:  
60 (D) OTHER INFORMATION: A at positions 4 and 9 is 2'-O-Methyl-2'-deoxyadenosine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: G at positions 5 and 6 is 2'-O-Methyl-2'-deoxyguanosine.  
65 (ix) FEATURE:  
(D) OTHER INFORMATION: C at position 7 is 2'-O-Methyl-2'-deoxycytidine.

## 100

- (ix) FEATURE:  
(D) OTHER INFORMATION: U at position 8 is 2'-O-Methyl-2'-deoxyuridine.
- 5 (ix) FEATURE:  
(D) OTHER INFORMATION: Nucleotide 40 is an inverted orientation T (3'-3'-linked).  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 102:  
CACAGGCUAC GGCACGTAGA GCATCACCAT GATCCTGTGT 40
- 10 (2) INFORMATION FOR SEQ ID NO: 103:  
(i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 40 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
15 (D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: DNA  
(ix) FEATURE:  
(D) OTHER INFORMATION: G at position 31 is 2'-O-Methyl-2'-deoxyguanosine.
- 20 (ix) FEATURE:  
(D) OTHER INFORMATION: A at position 32 is 2'-O-Methyl-2'-deoxyadenosine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: U at positions 33 and 36 is 2'-O-Methyl-2'-deoxyuridine.
- 25 (ix) FEATURE:  
(D) OTHER INFORMATION: C at positions 34 and 35 is 2'-O-Methyl-2'-deoxycytidine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: Nucleotide 40 is an inverted orientation T (3'-3'-linked).
- 30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 103:  
CACAGGCTAC GGCACGTAGA GCATCACCAT GAUCCUGTGT 40
- 35 (2) INFORMATION FOR SEQ ID NO: 104:  
(i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 40 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
40 (D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: DNA  
(ix) FEATURE:  
(D) OTHER INFORMATION: U at positions 17 and 24 is 2'-O-Methyl-2'-deoxyuridine.
- 45 (ix) FEATURE:  
(D) OTHER INFORMATION: A at position 23 is 2'-O-Methyl-2'-deoxyadenosine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: Nucleotide 40 is an inverted orientation T (3'-3'-linked).
- 50 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 104:  
CACAGGCTAC GGCACGUAGA GCAUCACCAT GATCCTGTGT 40
- 55 (2) INFORMATION FOR SEQ ID NO: 105:  
(i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 40 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
60 (ii) MOLECULAR TYPE: DNA  
(ix) FEATURE:  
(D) OTHER INFORMATION: G at positions 19 and 21 is 2'-O-Methyl-2'-deoxyguanosine.
- 65 (ix) FEATURE:  
(D) OTHER INFORMATION: A at position 20 is 2'-O-Methyl-2'-deoxyadenosine.

101

- (ix) FEATURE:  
(D) OTHER INFORMATION: C at position 22 is 2'-O-Methyl-2'-deoxycytidine.
- 5 (ix) FEATURE:  
(D) OTHER INFORMATION: Nucleotide 40 is an inverted orientation T (3'-3'-linked).  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 105:  
CACAGGCTAC GGCACGTAGA GCATCACCAT GATCCTGTGT 40
- 10 (2) INFORMATION FOR SEQ ID NO: 106:  
(i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 40 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
15 (D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: DNA  
(ix) FEATURE:  
(D) OTHER INFORMATION: A at position 20 is 2'-O-Methyl-2'-deoxyadenosine.
- 20 (ix) FEATURE:  
(D) OTHER INFORMATION: G at position 21 is 2'-O-Methyl-2'-deoxyguanosine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: C at position 22 is 2'-O-Methyl-2'-deoxycytidine.
- 25 (ix) FEATURE:  
(D) OTHER INFORMATION: Nucleotide 40 is an inverted orientation T (3'-3'-linked).  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 106:  
30 CACAGGCTAC GGCACGTAGA GCATCACCAT GATCCTGTGT 40
- (2) INFORMATION FOR SEQ ID NO: 107:  
(i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 40 base pairs  
35 (B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: DNA  
(ix) FEATURE:  
40 (D) OTHER INFORMATION: G at positions 12 and 16 is 2'-O-Methyl-2'-deoxyguanosine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: C at positions 13 and 15 is 2'-O-Methyl-2'-deoxycytidine.
- 45 (ix) FEATURE:  
(D) OTHER INFORMATION: A at position 14 is 2'-O-Methyl-2'-deoxyadenosine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: U at position 17 is 2'-O-Methyl-2'-deoxyuridine.
- 50 (ix) FEATURE:  
(D) OTHER INFORMATION: Nucleotide 40 is an inverted orientation T (3'-3'-linked).  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 107:  
55 CACAGGCTAC GGCACGUAGA GCATCACCAT GATCCTGTGT 40
- (2) INFORMATION FOR SEQ ID NO: 108:  
(i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 40 base pairs  
60 (B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: DNA  
(ix) FEATURE:  
65 (D) OTHER INFORMATION: C at positions 10 and 13 is 2'-O-Methyl-2'-deoxycytidine.

- (ix) FEATURE:  
(D) OTHER INFORMATION: G at positions 11 and 12 is 2'-O-Methyl-2'-deoxyguanosine.
- 5 (ix) FEATURE:  
(D) OTHER INFORMATION: A at position 14 is 2'-O-Methyl-2'-deoxyadenosine.
- (ix) FEATURE:  
(D) OTHER INFORMATION: Nucleotide 40 is an inverted orientation T (3'-3'-linked).
- 10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 108:  
CACAGGCTAC GGCACGTAGA GCATCACCAT GATCCTGTGT 40
- (2) INFORMATION FOR SEQ ID NO: 109:  
(i) SEQUENCE CHARACTERIZATION:  
15 (A) LENGTH: 40 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: DNA  
20 (ix) FEATURE:  
(D) OTHER INFORMATION: C at positions 1 and 3 is 2'-O-Methyl-2'-deoxycytidine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: A at positions 2 and 4 is 2'-O-Methyl-2'-deoxyadenosine.  
25 (ix) FEATURE:  
(D) OTHER INFORMATION: Nucleotide 40 is an inverted orientation T (3'-3'-linked).  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 109:  
30 CACAGGCTAC GGCACGTAGA GCATCACCAT GATCCTGTGT 40
- (2) INFORMATION FOR SEQ ID NO: 110:  
(i) SEQUENCE CHARACTERIZATION:  
35 (A) LENGTH: 40 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: DNA  
(ix) FEATURE:  
40 (D) OTHER INFORMATION: U at positions 36 and 38 is 2'-O-Methyl-2'-deoxyuridine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: G at positions 37 and 39 is 2'-O-Methyl-2'-deoxyguanosine.  
45 (ix) FEATURE:  
(D) OTHER INFORMATION: Nucleotide 40 is an inverted orientation T (3'-3'-linked).  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 110:  
50 CACAGGCTAC GGCACGTAGA GCATCACCAT GATCCUGUGT 40
- (2) INFORMATION FOR SEQ ID NO: 111:  
(i) SEQUENCE CHARACTERIZATION:  
55 (A) LENGTH: 40 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ix) FEATURE:  
(D) OTHER INFORMATION: C at position 7 is 2'-fluoro-2'-deoxycytidine.  
60 (ix) FEATURE:  
(D) OTHER INFORMATION: Nucleotide 40 is an inverted orientation T (3'-3'-linked).  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 111:  
65 CACAGGCTAC GGCACGTAGA GCATCACCAT GATCCTGTGT 40
- (2) INFORMATION FOR SEQ ID NO: 112:



103

- (i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 40 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (ix) FEATURE:  
(D) OTHER INFORMATION: C at position 22 is 2'-fluoro-2'-deoxycytidine.
- (ix) FEATURE:  
(D) OTHER INFORMATION: Nucleotide 40 is an inverted orientation T (3'-3'-linked).
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 112:  
CACAGGCTAC GGCACGTAGA GCATCACCAT GATCCTGTGT 40
- (2) INFORMATION FOR SEQ ID NO: 113:
- (i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 40 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (ix) FEATURE:  
(D) OTHER INFORMATION: C at position 25 is 2'-fluoro-2'-deoxycytidine.
- (ix) FEATURE:  
(D) OTHER INFORMATION: Nucleotide 40 is an inverted orientation T (3'-3'-linked).
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 113:  
CACAGGCTAC GGCACGTAGA GCATCACCAT GATCCTGTGT 40
- (2) INFORMATION FOR SEQ ID NO: 114:
- (i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 40 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (ix) FEATURE:  
(D) OTHER INFORMATION: C at position 34 is 2'-fluoro-2'-deoxycytidine.
- (ix) FEATURE:  
(D) OTHER INFORMATION: Nucleotide 40 is an inverted orientation T (3'-3'-linked).
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 114:  
CACAGGCTAC GGCACGTAGA GCATCACCAT GATCCTGTGT 40
- (2) INFORMATION FOR SEQ ID NO: 115:
- (i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 40 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (ix) FEATURE:  
(D) OTHER INFORMATION: C at position 35 is 2'-fluoro-2'-deoxycytidine.
- (ix) FEATURE:  
(D) OTHER INFORMATION: Nucleotide 40 is an inverted orientation T (3'-3'-linked).
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 115:  
CACAGGCTAC GGCACGTAGA GCATCACCAT GATCCTGTGT 40
- (2) INFORMATION FOR SEQ ID NO: 116:
- (i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 40 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (ix) FEATURE:

- (D) OTHER INFORMATION: U at position 8 is 2'-fluoro-2'-deoxyuridine.
- (ix) FEATURE:
- (D) OTHER INFORMATION: Nucleotide 40 is an inverted orientation T (3'-3'-linked).
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 116:  
CACAGGCUAC GGCACGTAGA GCATCACCAT GATCCTGTGT 40
- (2) INFORMATION FOR SEQ ID NO: 117:
- (i) SEQUENCE CHARACTERIZATION:
- (A) LENGTH: 40 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ix) FEATURE:
- (D) OTHER INFORMATION: U at position 17 is 2'-fluoro-2'-deoxyuridine.
- (ix) FEATURE:
- (D) OTHER INFORMATION: Nucleotide 40 is an inverted orientation T (3'-3'-linked).
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 117:  
CACAGGCTAC GGCACGUAGA GCATCACCAT GATCCTGTGT 40
- (2) INFORMATION FOR SEQ ID NO: 118:
- (i) SEQUENCE CHARACTERIZATION:
- (A) LENGTH: 40 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ix) FEATURE:
- (D) OTHER INFORMATION: U at position 24 is 2'-fluoro-2'-deoxyuridine.
- (ix) FEATURE:
- (D) OTHER INFORMATION: Nucleotide 40 is an inverted orientation T (3'-3'-linked).
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 118:  
CACAGGCTAC GGCACGTAGA GCAUCACCAT GATCCTGTGT 40
- (2) INFORMATION FOR SEQ ID NO: 119:
- (i) SEQUENCE CHARACTERIZATION:
- (A) LENGTH: 40 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ix) FEATURE:
- (D) OTHER INFORMATION: U at position 30 is 2'-fluoro-2'-deoxyuridine.
- (ix) FEATURE:
- (D) OTHER INFORMATION: Nucleotide 40 is an inverted orientation T (3'-3'-linked).
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 119:  
CACAGGCTAC GGCACGTAGA GCATCACCAU GATCCTGTGT 40
- (2) INFORMATION FOR SEQ ID NO: 120:
- (i) SEQUENCE CHARACTERIZATION:
- (A) LENGTH: 40 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ix) FEATURE:
- (D) OTHER INFORMATION: U at position 33 is 2'-fluoro-2'-deoxyuridine.
- (ix) FEATURE:
- (D) OTHER INFORMATION: Nucleotide 40 is an inverted orientation T (3'-3'-linked).
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 120:

105

CACAGGCTAC GGCACGTAGA GCATCACCAT GAUCCTGTGT

40

(2) INFORMATION FOR SEQ ID NO: 121:

(i) SEQUENCE CHARACTERIZATION:

(A) LENGTH: 40 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ix) FEATURE:

(D) OTHER INFORMATION: C at positions 7, 22, 25, 34 and 35 is 2'-fluoro-2'-deoxycytidine.

(ix) FEATURE:

(D) OTHER INFORMATION: U at positions 8, 17, 24, 33 and 36 is 2'-fluoro-2'-deoxyuridine.

(ix) FEATURE:

(D) OTHER INFORMATION: Nucleotide 40 is an inverted orientation T (3'-3'-linked).

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 121:

CACAGGCUAC GGCACGUAGA GCAUCACCAT GAUCCUGTGT

40

(2) INFORMATION FOR SEQ ID NO: 122:

(i) SEQUENCE CHARACTERIZATION:

(A) LENGTH: 40 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ix) FEATURE:

(D) OTHER INFORMATION: C at positions 10, 13, 27 and 28 is 2'-O-Methyl-2'-deoxycytidine.

(ix) FEATURE:

(D) OTHER INFORMATION: G at positions 11, 12, 37 and 39 is 2'-O-Methyl-2'-deoxyguanosine.

(ix) FEATURE:

(D) OTHER INFORMATION: A at positions 14, 26 and 29 is 2'-O-Methyl-2'-deoxyadenosine.

(ix) FEATURE:

(D) OTHER INFORMATION: C at position 34 is 2'-fluoro-2'-deoxycytidine.

(ix) FEATURE:

(D) OTHER INFORMATION: U at position 38 is 2'-O-Methyl-2'-deoxyuridine.

(ix) FEATURE:

(D) OTHER INFORMATION: Nucleotide 40 is an inverted orientation T (3'-3'-linked).

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 122:

CACAGGCTAC GGCACGTAGA GCATCACCAT GATCCTGUGT

40

(2) INFORMATION FOR SEQ ID NO: 123:

(i) SEQUENCE CHARACTERIZATION:

(A) LENGTH: 40 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ix) FEATURE:

(D) OTHER INFORMATION: C at positions 10, 13, 25, 27 and 28 is 2'-O-Methyl-2'-deoxycytidine.

(ix) FEATURE:

(D) OTHER INFORMATION: G at positions 11, 12, 37 and 39 is 2'-O-Methyl-2'-deoxyguanosine.

(ix) FEATURE:

(D) OTHER INFORMATION: A at positions 14, 26 and 29 is 2'-O-Methyl-2'-deoxyadenosine.

(ix) FEATURE:

(D) OTHER INFORMATION: C at position 34 is 2'-fluoro-2'-deoxycytidine.

(ix) FEATURE:

106

(D) OTHER INFORMATION: U at positions 36 and 38 is 2'-O-Methyl-2'-deoxyuridine.

(ix) FEATURE:

5 (D) OTHER INFORMATION: Nucleotide 40 is an inverted orientation T (3'-3'-linked).

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 123:

CACAGGCTAC GGCACGTAGA GCATCACCAT GATCCUGUGT

40

(2) INFORMATION FOR SEQ ID NO: 124:

10 (i) SEQUENCE CHARACTERIZATION:

(A) LENGTH: 40 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

15 (ix) FEATURE:

(D) OTHER INFORMATION: U at positions 8 and 24 is 2'-fluoro-2'-deoxyuridine.

(ix) FEATURE:

20 (D) OTHER INFORMATION: C at positions 10, 13, 27 and 28 is 2'-O-Methyl-2'-deoxycytidine.

(ix) FEATURE:

(D) OTHER INFORMATION: G at positions 11, 12, 37 and 39 is 2'-O-Methyl-2'-deoxyguanosine.

(ix) FEATURE:

25 (D) OTHER INFORMATION: A at positions 14, 26 and 29 is 2'-O-Methyl-2'-deoxyadenosine.

(ix) FEATURE:

(D) OTHER INFORMATION: C at positions 25, 34 and 35 is 2'-fluoro-2'-deoxycytidine.

30 (ix) FEATURE:

(D) OTHER INFORMATION: U at positions 36 and 38 is 2'-O-Methyl-2'-deoxyuridine.

(ix) FEATURE:

35 (D) OTHER INFORMATION: Nucleotide 40 is an inverted orientation T (3'-3'-linked).

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 124:

CACAGGCUAC GGCACGTAGA GCAUCACCAT GATCCUGUGT

40

(2) INFORMATION FOR SEQ ID NO: 125:

40 (i) SEQUENCE CHARACTERIZATION:

(A) LENGTH: 40 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

45 (ix) FEATURE:

(D) OTHER INFORMATION: C at positions 10, 13, 25, 27 and 28 is 2'-O-Methyl-2'-deoxycytidine.

(ix) FEATURE:

50 (D) OTHER INFORMATION: G at positions 11, 12, 37 and 39 is 2'-O-Methyl-2'-deoxyguanosine.

(ix) FEATURE:

(D) OTHER INFORMATION: A at positions 14, 26 and 29 is 2'-O-Methyl-2'-deoxyadenosine.

(ix) FEATURE:

55 (D) OTHER INFORMATION: C at positions 34 and 35 is 2'-fluoro-2'-deoxycytidine.

(ix) FEATURE:

(D) OTHER INFORMATION: U at positions 36 and 38 is 2'-O-Methyl-2'-deoxyuridine.

60 (ix) FEATURE:

(D) OTHER INFORMATION: Nucleotide 40 is an inverted orientation T (3'-3'-linked).

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 125:

CACAGGCTAC GGCACGTAGA GCATCACCAT GATCCUGUGT

40

65

(2) INFORMATION FOR SEQ ID NO: 126:

107

- (i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 40 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (ix) FEATURE:  
(D) OTHER INFORMATION: U at position 8 is 2'-fluoro-2'-deoxyuridine.
- (ix) FEATURE:  
(D) OTHER INFORMATION: C at positions 10, 13, 25, 27 and 28 is 2'-O-Methyl-2'-deoxycytidine.
- (ix) FEATURE:  
(D) OTHER INFORMATION: G at positions 11, 12, 37 and 39 is 2'-O-Methyl-2'-deoxyguanosine.
- (ix) FEATURE:  
(D) OTHER INFORMATION: A at positions 14, 26 and 29 is 2'-O-Methyl-2'-deoxyadenosine.
- (ix) FEATURE:  
(D) OTHER INFORMATION: C at position 34 is 2'-fluoro-2'-deoxycytidine.
- (ix) FEATURE:  
(D) OTHER INFORMATION: U at positions 36 and 38 is 2'-O-Methyl-2'-deoxyuridine.
- (ix) FEATURE:  
(D) OTHER INFORMATION: Nucleotide 40 is an inverted orientation T (3'-3'-linked).
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 126:  
CACAGGCUAC GGCACGTAGA GCATCACCAT GATCCUGUGT 40
- (2) INFORMATION FOR SEQ ID NO: 127:
- (i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 36 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (ix) FEATURE:  
(D) OTHER INFORMATION: U at positions 6 and 22 is 2'-fluoro-2'-deoxyuridine.
- (ix) FEATURE:  
(D) OTHER INFORMATION: C at positions 8, 11, 25 and 26 is 2'-O-Methyl-2'-deoxycytidine.
- (ix) FEATURE:  
(D) OTHER INFORMATION: G at positions 9, 10, and 35 is 2'-O-Methyl-2'-deoxyguanosine.
- (ix) FEATURE:  
(D) OTHER INFORMATION: A at positions 12, 24 and 27 is 2'-O-Methyl-2'-deoxyadenosine.
- (ix) FEATURE:  
(D) OTHER INFORMATION: C at positions 23, 32 and 33 is 2'-fluoro-2'-deoxycytidine.
- (ix) FEATURE:  
(D) OTHER INFORMATION: U at position 34 is 2'-O-Methyl-2'-deoxyuridine.
- (ix) FEATURE:  
(D) OTHER INFORMATION: Nucleotide 36 is an inverted orientation T (3'-3'-linked).
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 127:  
CAGGCUACGG CACGTAGAGC AUCACCATGA TCCUGT 36
- (2) INFORMATION FOR SEQ ID NO: 128:
- (i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 34 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (ix) FEATURE:

108

- (D) OTHER INFORMATION: U at positions 6 and 20 is 2'-fluoro-2'-deoxyuridine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: C at positions 7, 10, 23 and 24 is  
5 2'-O-Methyl-2'-deoxycytidine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: G at positions 8, 9, and 33 is 2'-O-Methyl-2'-deoxyguanosine.  
(ix) FEATURE:  
10 (D) OTHER INFORMATION: A at positions 11, 22 and 25 is 2'-O-Methyl-2'-deoxyadenosine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: C at positions 21, 30 and 31 is 2'-fluoro-2'-deoxycytidine.  
15 (ix) FEATURE:  
(D) OTHER INFORMATION: U at position 32 is 2'-O-Methyl-2'-deoxyuridine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: Nucleotide 34 is an inverted  
20 orientation T (3'-3'-linked).  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 128:  
CAGGCUCGGC ACGAGAGCAU CACCATGATC CUGT 34
- (2) INFORMATION FOR SEQ ID NO: 129:  
25 (i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 32 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
30 (ix) FEATURE:  
(D) OTHER INFORMATION: U at positions 6 and 20 is 2'-fluoro-2'-deoxyuridine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: C at positions 7, 10, 22 and 23 is  
35 2'-O-Methyl-2'-deoxycytidine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: G at positions 8, 9, and 31 is 2'-O-Methyl-2'-deoxyguanosine.  
(ix) FEATURE:  
40 (D) OTHER INFORMATION: A at positions 11 and 24 is 2'-O-Methyl-2'-deoxyadenosine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: C at positions 21, 28 and 29 is 2'-fluoro-2'-deoxycytidine.  
45 (ix) FEATURE:  
(D) OTHER INFORMATION: U at position 30 is 2'-O-Methyl-2'-deoxyuridine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: Nucleotide 32 is an inverted  
50 orientation T (3'-3'-linked).  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 129:  
CAGGCUCGGC ACGAGAGCAU CCCAGATCCU GT 32
- (2) INFORMATION FOR SEQ ID NO: 130:  
55 (i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 36 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
60 (ix) FEATURE:  
(D) OTHER INFORMATION: C at positions 8, 11, 23, 25, 26, 32 and 33 is 2'-O-Methyl-2'-deoxycytidine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: G at positions 9, 10, and 35 is 2'-O-Methyl-2'-deoxyguanosine.  
65 (ix) FEATURE:

109

(D) OTHER INFORMATION: A at positions 12, 24 and 27 is 2'-O-Methyl-2'-deoxyadenosine.

(ix) FEATURE:

5 (D) OTHER INFORMATION: U at position 6 is 2'-fluoro-2'-deoxyuridine.

(ix) FEATURE:

(D) OTHER INFORMATION: U at positions 22 and 34 is 2'-O-Methyl-2'-deoxyuridine.

(ix) FEATURE:

10 (D) OTHER INFORMATION: Nucleotide 36 is an inverted orientation T (3'-3'-linked).

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 130:

CAGGCUACGG CACGTAGAGC AUCACCATGA TCCUGT 36

15 (2) INFORMATION FOR SEQ ID NO: 131:

(i) SEQUENCE CHARACTERIZATION:

(A) LENGTH: 36 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

20 (D) TOPOLOGY: linear

(ix) FEATURE:

(D) OTHER INFORMATION: C at positions 5, 8, 11, 23, 25, 26, 32 and 33 is 2'-O-Methyl-2'-deoxycytidine.

(ix) FEATURE:

25 (D) OTHER INFORMATION: U at positions 6, 22 and 34 is 2'-O-Methyl-2'-deoxyuridine.

(ix) FEATURE:

(D) OTHER INFORMATION: A at positions 7, 12, 24 and 27 is 2'-O-Methyl-2'-deoxyadenosine.

30 (ix) FEATURE:

(D) OTHER INFORMATION: G at positions 9, 10, and 35 is 2'-O-Methyl-2'-deoxyguanosine.

(ix) FEATURE:

35 (D) OTHER INFORMATION: Nucleotide 36 is an inverted orientation T (3'-3'-linked).

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 131:

CAGGCUACGG CACGTAGAGC AUCACCATGA TCCUGT 36

40 (2) INFORMATION FOR SEQ ID NO: 132:

(i) SEQUENCE CHARACTERIZATION:

(A) LENGTH: 36 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

45 (D) TOPOLOGY: linear

(ix) FEATURE:

(D) OTHER INFORMATION: C at positions 5, 8, 11, 23, 25, 26, 32 and 33 is 2'-O-Methyl-2'-deoxycytidine.

(ix) FEATURE:

50 (D) OTHER INFORMATION: U at positions 6, 22 and 34 is 2'-O-Methyl-2'-deoxyuridine.

(ix) FEATURE:

(D) OTHER INFORMATION: A at positions 12, 18, 24 and 27 is 2'-O-Methyl-2'-deoxyadenosine.

(ix) FEATURE:

55 (D) OTHER INFORMATION: G at positions 9, 10, and 35 is 2'-O-Methyl-2'-deoxyguanosine.

(ix) FEATURE:

(D) OTHER INFORMATION: Nucleotide 36 is an inverted orientation T (3'-3'-linked).

60 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 132:

CAGGCUACGG CACGTAGAGC AUCACCATGA TCCUGT 36

(2) INFORMATION FOR SEQ ID NO: 133:

(i) SEQUENCE CHARACTERIZATION:

65 (A) LENGTH: 36 base pairs

(B) TYPE: nucleic acid

110

- (C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ix) FEATURE:  
(D) OTHER INFORMATION: C at positions 5, 8, 11, 23, 25, 26,  
5 32 and 33 is 2'-O-Methyl-2'-deoxycytidine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: U at positions 6, 22 and 34 is 2'-O-Methyl-2'-deoxyuridine.  
(ix) FEATURE:  
10 (D) OTHER INFORMATION: G at positions 9, 10, 19 and 35 is 2'-O-Methyl-2'-deoxyguanosine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: A at positions 12, 24 and 27 is 2'-O-Methyl-2'-deoxyadenosine.  
15 (ix) FEATURE:  
(D) OTHER INFORMATION: Nucleotide 36 is an inverted orientation T (3'-3'-linked).  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 133:  
CAGGCUACGG CACGTAGAGC AUCACCATGA TCCUGT 36  
20  
(2) INFORMATION FOR SEQ ID NO: 134:  
(i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 36 base pairs  
(B) TYPE: nucleic acid  
25 (C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ix) FEATURE:  
(D) OTHER INFORMATION: U at positions 6 and 22 is 2'-fluoro-2'-deoxyuridine.  
30 (ix) FEATURE:  
(D) OTHER INFORMATION: C at positions 8, 11, 25 and 26 is 2'-O-Methyl-2'-deoxycytidine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: G at positions 9, 10, 19 and 35 is  
35 2'-O-Methyl-2'-deoxyguanosine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: A at positions 12, 24 and 27 is 2'-O-Methyl-2'-deoxyadenosine.  
(ix) FEATURE:  
40 (D) OTHER INFORMATION: C at positions 23, 32 and 33 is 2'-fluoro-2'-deoxycytidine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: U at position 34 is 2'-O-Methyl-2'-deoxyuridine.  
45 (ix) FEATURE:  
(D) OTHER INFORMATION: Nucleotide 36 is an inverted orientation T (3'-3'-linked).  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 134:  
CAGGCUACGG CACGTAGAGC AUCACCATGA TCCUGT 36  
50  
(2) INFORMATION FOR SEQ ID NO: 135:  
(i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 36 base pairs  
(B) TYPE: nucleic acid  
55 (C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ix) FEATURE:  
(D) OTHER INFORMATION: U at positions 6 and 22 is 2'-fluoro-2'-deoxyuridine.  
60 (ix) FEATURE:  
(D) OTHER INFORMATION: C at positions 8, 11, 20, 25 and 26 is 2'-O-Methyl-2'-deoxycytidine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: G at positions 9, 10, 19 and 35 is  
65 2'-O-Methyl-2'-deoxyguanosine.  
(ix) FEATURE:



111

- (D) OTHER INFORMATION: A at positions 12, 24 and 27 is 2'-O-Methyl-2'-deoxyadenosine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: C at positions 23, 32 and 33 is 2'-fluoro-2'-deoxycytidine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: U at position 34 is 2'-O-Methyl-2'-deoxyuridine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: Nucleotide 36 is an inverted orientation T (3'-3'-linked).  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 135:  
CAGGCUACGG CACGTAGAGC AUCACCATGA TCCUGT 36
- 15 (2) INFORMATION FOR SEQ ID NO: 136:  
(i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 36 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ix) FEATURE:  
(D) OTHER INFORMATION: U at positions 6 and 22 is 2'-fluoro-2'-deoxyuridine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: C at positions 8, 11, 20, 25 and 26 is 2'-O-Methyl-2'-deoxycytidine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: G at positions 9, 10, 17, 19 and 35 is 2'-O-Methyl-2'-deoxyguanosine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: A at positions 12, 24 and 27 is 2'-O-Methyl-2'-deoxyadenosine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: C at positions 23, 32 and 33 is 2'-fluoro-2'-deoxycytidine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: U at position 34 is 2'-O-Methyl-2'-deoxyuridine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: Nucleotide 36 is an inverted orientation T (3'-3'-linked).  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 136:  
CAGGCUACGG CACGTAGAGC AUCACCATGA TCCUGT 36
- 45 (2) INFORMATION FOR SEQ ID NO: 137:  
(i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 36 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ix) FEATURE:  
(D) OTHER INFORMATION: U at positions 6 and 22 is 2'-fluoro-2'-deoxyuridine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: C at positions 8, 11, 25 and 26 is 2'-O-Methyl-2'-deoxycytidine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: G at positions 9, 10, 17, 19 and 35 is 2'-O-Methyl-2'-deoxyguanosine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: A at positions 12, 24 and 27 is 2'-O-Methyl-2'-deoxyadenosine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: C at positions 23, 32 and 33 is 2'-fluoro-2'-deoxycytidine.  
(ix) FEATURE:

- (D) OTHER INFORMATION: U at position 34 is 2'-O-Methyl-2'-deoxyuridine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: Nucleotide 36 is an inverted orientation T (3'-3'-linked).  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 137:  
CAGGCUACGG CACGTAGAGC AUCACCATGA TCCUGT 36
- (2) INFORMATION FOR SEQ ID NO: 138:  
(i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 36 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ix) FEATURE:  
(D) OTHER INFORMATION: U at positions 6 and 22 is 2'-fluoro-2'-deoxyuridine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: A at positions 7, 12, 24 and 27 is 2'-O-Methyl-2'-deoxyadenosine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: C at positions 8, 11, 25 and 26 is 2'-O-Methyl-2'-deoxycytidine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: G at positions 9, 10, 17, 19 and 35 is 2'-O-Methyl-2'-deoxyguanosine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: C at positions 23, 32 and 33 is 2'-fluoro-2'-deoxycytidine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: U at position 34 is 2'-O-Methyl-2'-deoxyuridine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: Nucleotide 36 is an inverted orientation T (3'-3'-linked).  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 138:  
CAGGCUACGG CACGTAGAGC AUCACCATGA TCCUGT 36
- (2) INFORMATION FOR SEQ ID NO: 139:  
(i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 32 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ix) FEATURE:  
(D) OTHER INFORMATION: U at positions 6 and 20 is 2'-fluoro-2'-deoxyuridine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: C at position 8 is 2'-O-Methyl-2'-deoxycytidine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: G at positions 9, 17, and 31 is 2'-O-Methyl-2'-deoxyguanosine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: S at positions 10 and 23 is a hexaethyleneglycol spacer.  
(ix) FEATURE:  
(D) OTHER INFORMATION: C at positions 21, 28 and 29 is 2'-fluoro-2'-deoxycytidine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: A at position 22 is 2'-O-Methyl-2'-deoxyadenosine.  
(ix) FEATURE:  
(D) OTHER INFORMATION: U at position 30 is 2'-O-Methyl-2'-deoxyuridine.  
(ix) FEATURE:

113

(D) OTHER INFORMATION: Nucleotide 32 is an inverted orientation T (3'-3'-linked).

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 139:  
CAGGCUACGS CGTAGAGCAU CASTGATCCU GT

32

(2) INFORMATION FOR SEQ ID NO: 140:

(i) SEQUENCE CHARACTERIZATION:

(A) LENGTH: 32 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ix) FEATURE:

(D) OTHER INFORMATION: U at positions 6 and 20 is 2'-fluoro-2'-deoxyuridine.

(ix) FEATURE:

(D) OTHER INFORMATION: C at position 8 is 2'-O-Methyl-2'-deoxycytidine.

(ix) FEATURE:

(D) OTHER INFORMATION: G at positions 9, 15, 17, and 31 is 2'-O-Methyl-2'-deoxyguanosine.

(ix) FEATURE:

(D) OTHER INFORMATION: S at positions 10 and 23 is a hexaethyleneglycol spacer.

(ix) FEATURE:

(D) OTHER INFORMATION: C at positions 21, 28 and 29 is 2'-fluoro-2'-deoxycytidine.

(ix) FEATURE:

(D) OTHER INFORMATION: A at position 22 is 2'-O-Methyl-2'-deoxyadenosine.

(ix) FEATURE:

(D) OTHER INFORMATION: U at position 30 is 2'-O-Methyl-2'-deoxyuridine.

(ix) FEATURE:

(D) OTHER INFORMATION: Nucleotide 32 is an inverted orientation T (3'-3'-linked).

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 140:  
CAGGCUACGS CGTAGAGCAU CASTGATCCU GT

32

(2) INFORMATION FOR SEQ ID NO: 141:

(i) SEQUENCE CHARACTERIZATION:

(A) LENGTH: 36 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ix) FEATURE:

(D) OTHER INFORMATION: Nucleotide 36 is an inverted orientation T (3'-3'-linked).

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 141:  
CAGGCTACGG CACGTAGAGC ATCACCATGA TCCTGT

36

(2) INFORMATION FOR SEQ ID NO: 142:

(i) SEQUENCE CHARACTERIZATION:

(A) LENGTH: 32 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ix) FEATURE:

(D) OTHER INFORMATION: U at positions 6, 20 and 30 is 2'-fluoro-2'-deoxyuridine.

(ix) FEATURE:

(D) OTHER INFORMATION: C at positions 8, 21, 28 and 29 is 2'-fluoro-2'-deoxycytidine.

(ix) FEATURE:

(D) OTHER INFORMATION: G at positions 9, 15, 17, and 31 is 2'-O-Methyl-2'-deoxyguanosine.

(ix) FEATURE:

114

(D) OTHER INFORMATION: S at positions 10 and 23 is a hexaethyleneglycol spacer.

(ix) FEATURE:

5 (D) OTHER INFORMATION: A at position 22 is 2'-O-Methyl-2'-deoxyadenosine.

(ix) FEATURE:

(D) OTHER INFORMATION: Nucleotide 32 is an inverted orientation T (3'-3'-linked).

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 142:

10 CAGGCUACGS CGTAGAGCAU CASTGATCCU GT 32

(2) INFORMATION FOR SEQ ID NO: 143:

(i) SEQUENCE CHARACTERIZATION:

15 (A) LENGTH: 32 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ix) FEATURE:

20 (D) OTHER INFORMATION: U at positions 6, 20 and 30 is 2'-fluoro-2'-deoxyuridine.

(ix) FEATURE:

(D) OTHER INFORMATION: C at positions 8, 21, 28 and 29 is 2'-fluoro-2'-deoxycytidine.

(ix) FEATURE:

25 (D) OTHER INFORMATION: G at positions 9, 15, 17, and 31 is 2'-O-Methyl-2'-deoxyguanosine.

(ix) FEATURE:

(D) OTHER INFORMATION: N at positions 10 and 23 is a hexaethyleneglycol spacer.

30 (ix) FEATURE:

(D) OTHER INFORMATION: A at position 22 is 2'-O-Methyl-2'-deoxyadenosine.

(ix) FEATURE:

35 (D) OTHER INFORMATION: Nucleotide 32 is an inverted orientation T (3'-3'-linked).

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 143:

CAGGCUACGN CGTAGAGCAU CANTGATCCU GT 32

(2) INFORMATION FOR SEQ ID NO: 144:

40 (i) SEQUENCE CHARACTERIZATION:

(A) LENGTH: 32 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

45 (ix) FEATURE:

(D) OTHER INFORMATION: G at positions 3, 4, 12, and 25 is 2'-O-Methyl-2'-deoxyguanosine.

(ix) FEATURE:

50 (D) OTHER INFORMATION: U at positions 6, 20 and 27 is 2'-fluoro-2'-deoxyuridine.

(ix) FEATURE:

(D) OTHER INFORMATION: N at positions 10 and 23 is a hexaethyleneglycol spacer.

(ix) FEATURE:

55 (D) OTHER INFORMATION: C at positions 11, 18, 21 and 29 is 2'-fluoro-2'-deoxycytidine.

(ix) FEATURE:

(D) OTHER INFORMATION: A at position 16 is 2'-O-Methyl-2'-deoxyadenosine.

60 (ix) FEATURE:

(D) OTHER INFORMATION: Nucleotide 32 is an inverted orientation T (3'-3'-linked).

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 144:

65 CAGGCUACGN CGTAGAGCAU CANTGAUCCT GT 32

(2) INFORMATION FOR SEQ ID NO: 145:

115

- (i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 32 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (ix) FEATURE:  
(D) OTHER INFORMATION: C at positions 4, 8, 21 and 29 is 2'-fluoro-2'-deoxycytidine.
- (ix) FEATURE:  
(D) OTHER INFORMATION: U at positions 6, 20 and 30 is 2'-fluoro-2'-deoxyuridine.
- (ix) FEATURE:  
(D) OTHER INFORMATION: G at positions 5, 9, 17, and 31 is 2'-O-Methyl-2'-deoxyguanosine.
- (ix) FEATURE:  
(D) OTHER INFORMATION: A at position 22 is 2'-O-Methyl-2'-deoxyadenosine.
- (ix) FEATURE:  
(D) OTHER INFORMATION: N at positions 10 and 23 is a hexaethylene glycol phosphoramidite.
- (ix) FEATURE:  
(D) OTHER INFORMATION: Nucleotide 32 is an inverted orientation T (3'-3'-linked).
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 145:  
CAGCGUACGN CGTACCGATU CANTGAAGCU GT 32
- (2) INFORMATION FOR SEQ ID NO: 146:  
(i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 32 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (ix) FEATURE:  
(D) OTHER INFORMATION: U at positions 6, 20 and 30 is 2'-fluoro-2'-deoxyuridine.
- (ix) FEATURE:  
(D) OTHER INFORMATION: C at positions 8, 21, 28, and 29 is 2'-fluoro-2'-deoxycytidine.
- (ix) FEATURE:  
(D) OTHER INFORMATION: G at positions 9, 15, 17, and 31 is 2'-O-Methyl-2'-deoxyguanosine.
- (ix) FEATURE:  
(D) OTHER INFORMATION: A at position 22 is 2'-O-Methyl-2'-deoxyadenosine.
- (ix) FEATURE:  
(D) OTHER INFORMATION: N at positions 10 and 23 is a hexaethylene glycol phosphoramidite.
- (ix) FEATURE:  
(D) OTHER INFORMATION: Nucleotide 32 is an inverted orientation T (3'-3'-linked).
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 146:  
CAGGCUACGN CGTAGAGCAU CANTGATCCU GT 32
- (2) INFORMATION FOR SEQ ID NO: 147:  
(i) SEQUENCE CHARACTERIZATION:  
(A) LENGTH: 32 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (ix) FEATURE:  
(D) OTHER INFORMATION: C at positions 4, 8, 21 and 29 is 2'-fluoro-2'-deoxycytidine.
- (ix) FEATURE:  
(D) OTHER INFORMATION: U at positions 6, 20 and 30 is 2'-fluoro-2'-deoxyuridine.
- (ix) FEATURE:

116

(D) OTHER INFORMATION: G at positions 5, 9, 17, and 31 is 2'-O-Methyl-2'-deoxyguanosine.

(ix) FEATURE:

5 (D) OTHER INFORMATION: A at position 22 is 2'-O-Methyl-2'-deoxyadenosine.

(ix) FEATURE:

(D) OTHER INFORMATION: N at positions 10 and 23 is a hexaethylene glycol phosphoramidite.

(ix) FEATURE:

10 (D) OTHER INFORMATION: Nucleotide 32 is an inverted orientation T (3'-3'-linked).

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 147:

CAGCGUACGN CGTACCGATU CANTGAAGCU GT 32

15 (2) INFORMATION FOR SEQ ID NO: 148:

(i) SEQUENCE CHARACTERIZATION:

(A) LENGTH: 32 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

20 (D) TOPOLOGY: linear

(ix) FEATURE:

(D) OTHER INFORMATION: U at positions 6, 20 and 30 is 2'-fluoro-2'-deoxyuridine.

(ix) FEATURE:

25 (D) OTHER INFORMATION: C at positions 8, 21, 28 and 29 is 2'-fluoro-2'-deoxycytidine.

(ix) FEATURE:

(D) OTHER INFORMATION: G at positions 9, 15, 17, and 31 is 2'-O-Methyl-2'-deoxyguanosine.

30 (ix) FEATURE:

(D) OTHER INFORMATION: S at positions 10 and 23 is a hexaethyleneglycol spacer.

(ix) FEATURE:

35 (D) OTHER INFORMATION: A at position 22 is 2'-O-Methyl-2'-deoxyadenosine.

(ix) FEATURE:

(D) OTHER INFORMATION: Nucleotide 32 is an inverted orientation T (3'-3'-linked).

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 148:

40 CAGGCUACGS CGTAGAGCAU CASTGATCCU GT 32

(2) INFORMATION FOR SEQ ID NO: 149:

(i) SEQUENCE CHARACTERIZATION:

(A) LENGTH: 32 base pairs

45 (B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ix) FEATURE:

50 (D) OTHER INFORMATION: C at positions 4, 8, 21 and 29 is 2'-fluoro-2'-deoxycytidine.

(ix) FEATURE:

(D) OTHER INFORMATION: G at positions 5, 9, 17, and 31 is 2'-O-Methyl-2'-deoxyguanosine.

(ix) FEATURE:

55 (D) OTHER INFORMATION: U at positions 6, 20 and 30 is 2'-fluoro-2'-deoxyuridine.

(ix) FEATURE:

(D) OTHER INFORMATION: N at positions 10 and 23 is a hexaethyleneglycol spacer.

60 (ix) FEATURE:

(D) OTHER INFORMATION: A at position 22 is 2'-O-Methyl-2'-deoxyadenosine.

(ix) FEATURE:

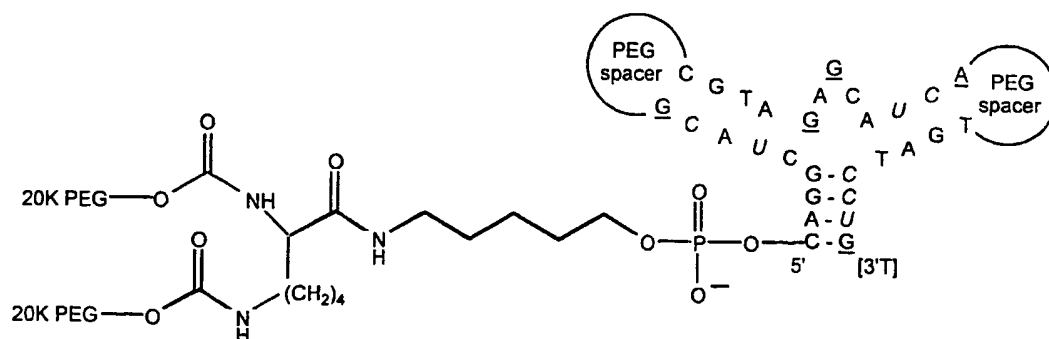
65 (D) OTHER INFORMATION: Nucleotide 32 is an inverted orientation T (3'-3'-linked).

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 149:

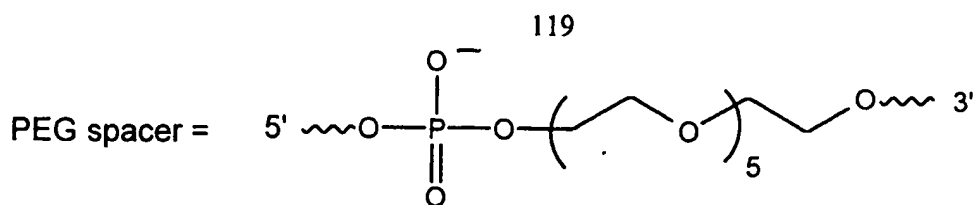
CAGCGUACGN CGTACCGATU CANTGAAGCU GT

**WE CLAIM:**

- 5           1.       A Complex comprised of a PDGF Nucleic Acid Ligand and a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound.
2.       The Complex of Claim 1 further comprising a Linker between said Ligand and said Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound.
- 10          3.       Claim 1 said Ligand comprises a Linker.
4.       The Complex of Claim 1 wherein said Non-Immunogenic, High Molecular Weight Compound is a Polyalkylene Glycol.
- 15          5.       The Complex of claim 4 wherein said Polyalkylene Glycol is polyethylene glycol.
6.       The Complex of claim 5 wherein said polyethylene glycol has a molecular weight of about between 10-80 K.
- 20          7.       The Complex of claim 6 wherein said polyethylene glycol has a molecular weight of about between 20-45 K.
- 25          8.       The Complex of claim 7 wherein said Complex is







9. The Complex of claim 1 wherein said Lipophilic Compound is a glycerol lipid.
- 5 10. A Lipid Construct comprising the Complex of claim 1.
11. The Complex of claim 1 wherein said PDGF Nucleic Acid Ligand was identified from a Candidate Mixture of Nucleic Acids according to the method comprising:
  - a) contacting the Candidate Mixture with PDGF, wherein the Nucleic Acids
  - 10 having an increased affinity to PDGF relative to the Candidate Mixture may be partitioned from the remainder of the Candidate Mixture;
  - b) partitioning the increased affinity Nucleic Acids from the remainder of the Candidate Mixture; and
  - c) amplifying the increased affinity Nucleic Acids to yield a mixture of
  - 15 Nucleic Acids enriched for Nucleic Acids having an increased affinity for PDGF; whereby Nucleic Acid Ligands of PDGF are produced.
12. The Complex of claim 11 wherein the method further comprises repeating steps b) and c).
- 20 13. A method for treating a PDGF mediated disease or medical condition comprising administering a pharmaceutically effective amount of a Complex comprised of a PDGF Nucleic Acid Ligand and a Non-Immunogenic, High Molecular Weight Compound or a Lipophilic Compound.
- 25 14. The Complex of Claim 13 further comprising a Linker between said Ligand and said Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound.
15. Claim 13 said Ligand comprises a Linker.

120

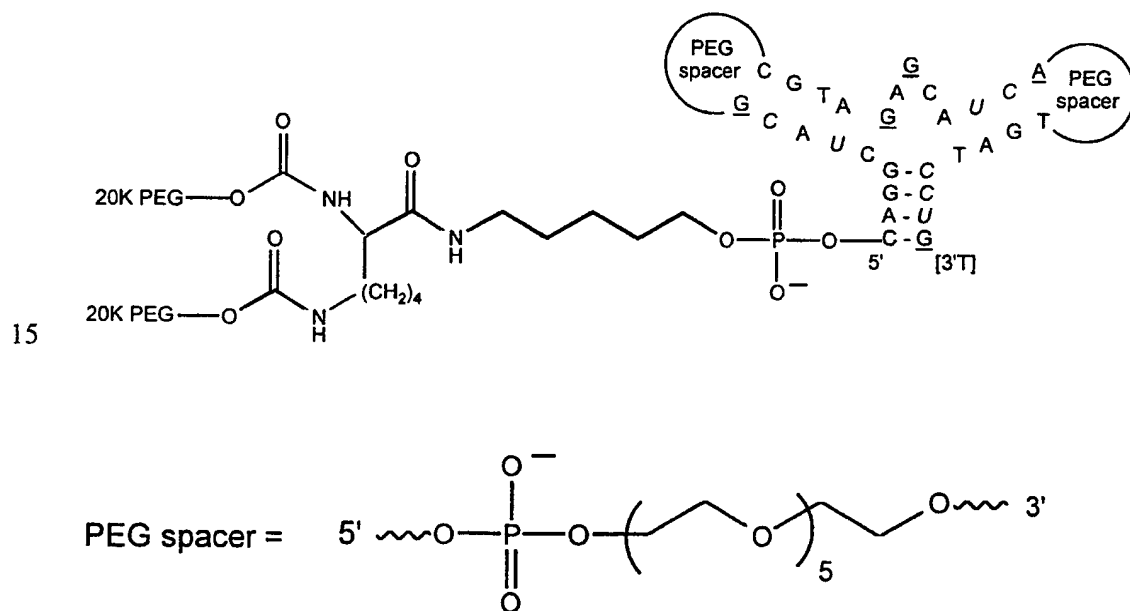
16. The method of claim 13 wherein said Non-Immunogenic, High Molecular Weight Compound is a Polyalkylene Glycol.

17. The method of claim 16 wherein said Polyalkylene Glycol is polyethylene glycol.

18. The method of claim 17 wherein said polyethylene glycol has a molecular weight of about between 10-80 K.

19. The method of claim 17 wherein said polyethylene glycol has a molecular weight of about 20-45 K.

20. The method of claim 16 wherein said Complex has the structure



21. The method of claim 11 wherein said Lipophilic Compound is a glycerol lipid.

22. The method of claim 21 wherein said Complex is further associated with a lipid construct.

23. A method for the preparation of a Complex comprised of a PDGF Nucleic Acid Ligand and a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound, said method comprising:

- a) identifying a PDGF Nucleic Acid Ligand from a Candidate Mixture of Nucleic Acids by the method comprising:
  - b) contacting the Candidate Mixture with PDGF, wherein Nucleic Acids having an increased affinity to PDGF relative to the Candidate Mixture may be partitioned from the remainder of the Candidate Mixture;
  - c) partitioning the increased affinity PDGF Nucleic Acids from the remainder of the Candidate Mixture;
  - e) amplifying the increased affinity PDGF Nucleic Acids to yield a ligand-enriched mixture of Nucleic Acids; and
  - f) associating said identified PDGF Nucleic Acid Ligand with a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound.

24. The method of claim 23 wherein said Complex is further associated with a Lipid Construct.

25. The method of claim 24 wherein said Lipid Construct is a Liposome.

26. The method of claim 25 wherein said Complex is comprised of a PDGF Nucleic Acid Ligand and a Lipophilic Compound and wherein said Complex is passively associated with the bilayer of said Liposomes by the method comprising the steps of:

- a) forming a liposome; and
- b) mixing said Complex comprised of a Nucleic Acid Ligand and a Lipophilic Compound with the Liposomes of step a) whereby the Nucleic Acid Ligand Component of said Complex becomes associated with the bilayer of the Liposome and projects from the exterior of the Lipid bilayer.

27. The method of Claim 26 wherein said Complex further comprises a Linker between said Ligand and said Lipophilic Compound.

28. The method of claim 26 wherein said Ligand comprises a Linker.

29. A method for improving the pharmacokinetic properties of a PDGF Nucleic Acid Ligand comprising:

5 covalently linking said PDGF Nucleic Acid Ligand with a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound to form a Complex comprised of a PDGF Nucleic Acid Ligand and a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound; and administering said Complex to a patient.

10 30. A method for targeting a therapeutic or diagnostic agent to a specific predetermined biological target that is expressing PDGF in a patient comprising:

covalently linking said therapeutic or diagnostic agent with a Complex comprised of a PDGF Nucleic Acid Ligand and a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound, and administering said Complex to a patient.

15

31. A method of inhibiting PDGF mediated angiogenesis comprising:

covalently linking a PDGF Nucleic Acid Ligand to a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound to form a Complex; and administering said Complex to a patient.

20

32. A method of inhibiting the growth of tumors comprising:

covalently linking a PDGF Nucleic Acid Ligand to a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound to form a Complex; and administering said Complex to a patient.

25

33. The method of claim 32 wherein said tumors or cells or tissues surrounding said tumors are expressing PDGF.

34. The method of claim 32 wherein said tumors or cells or tissues surrounding  
30 said tumors are expressing PDGF receptors.

35. A method of inhibiting fibrosis comprising:

covalently linking a PDGF Nucleic Acid Ligand to a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound to form a Complex; and administering said Complex to a patient.

5           36.    The method of Claim 35 wherein said Complex further comprises a Linker between said Ligand and said Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound.

10           37.    The method of claim 35 wherein said Ligand comprises a Linker.

          38.    The method of claim 35 wherein said fibrosis is kidney fibrosis.

          39.    The method of claim 35 wherein said fibrosis is lung fibrosis.

15           40.    The method of claim 35 wherein said fibrosis is bone marrow fibrosis.

          41.    The method of claim 35 wherein said fibrosis is radiation treatment-associated fibrosis.

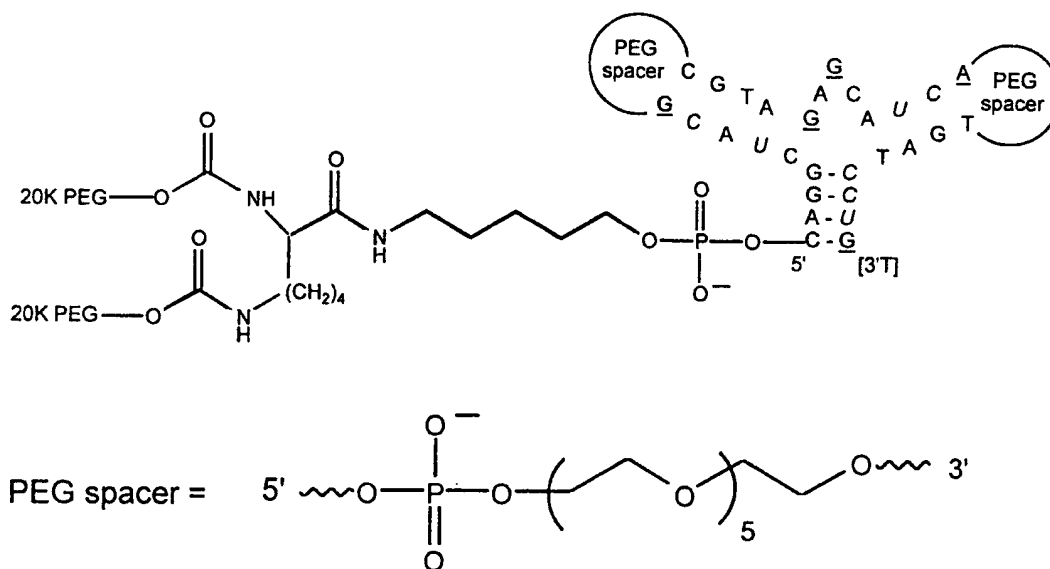
20           42.    The method of claim 36 wherein said Non-Immunogenic, High Molecular Weight Compound is a polyalkylene glycol.

          43.    The method of claim 42 wherein said polyalkylene glycol is polyethylene glycol.

25           44.    The method of claim 43 wherein said polyethylene glycol has a molecular weight of about between 10-80k.

          45.    The method of claim 43 wherein said polyethylene glycol has a molecular weight of about between 20-45k.

30           46.    The method of claim 45 wherein said complex has the structure



5

47. A method of inhibiting restenosis comprising:

covalently linking a PDGF Nucleic Acid Ligand to a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound to form a Complex; and administering said Complex to a patient.

10

48. The method of Claim 47 wherein said Complex further comprises a Linker between said Ligand and said Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound.

15

49. The method of claim 47 wherein said Ligand comprises a Linker.

50. The method of claim 47 wherein said Non-Immunogenic, High Molecular Weight Compound is a polyalkylene glycol.

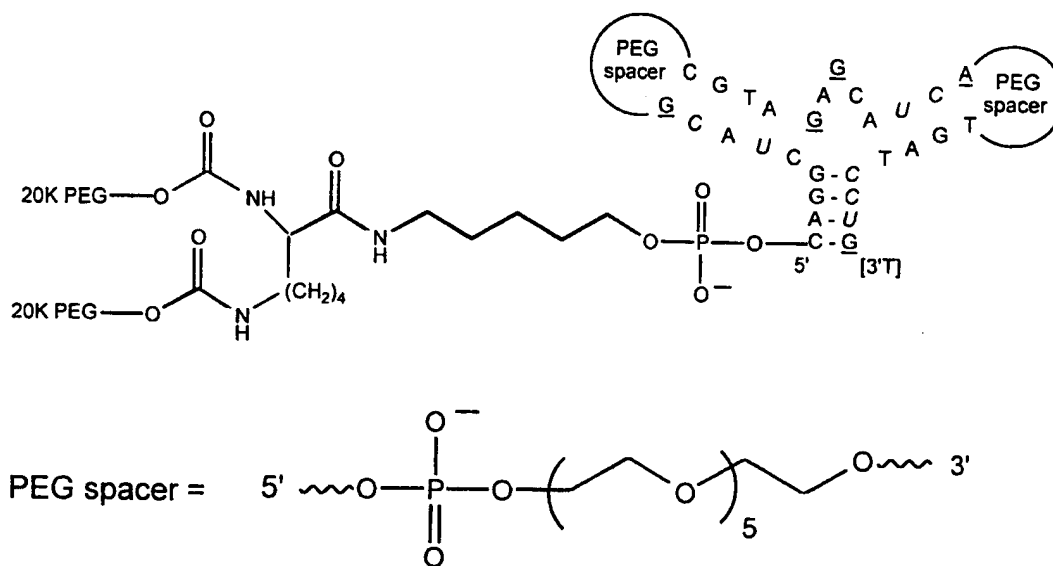
20

51. The method of claim 50 wherein said polyalkylene glycol is polyethylene glycol.

52. The method of claim 51 wherein said polyethylene glycol has a molecular weight of about between 10-80k.

53. The method of claim 51 wherein said polyethylene glycol has a molecular weight of about between 20-45k.

5 54. The method of claim 53 wherein said complex has the structure

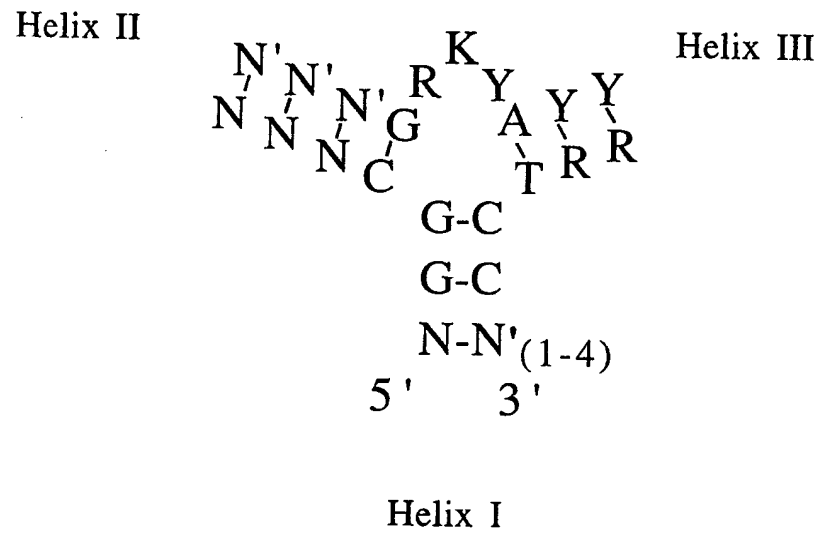


10

55. The method of claim 47 wherein said restenosis is in-stent restenosis.

56. The method of claim 47 wherein said restenosis is in a coronary artery.

15 57. The method of claim 47 wherein said restenosis is in a non-coronary vessel.



SEQ ID NO: 82

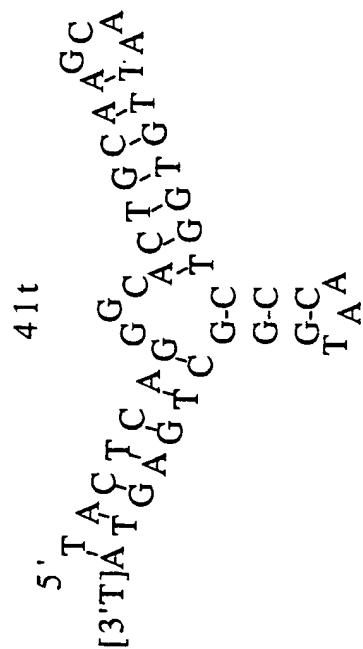
FIGURE 1





FIGURE 2B

SEQ ID NO: 84



3/26

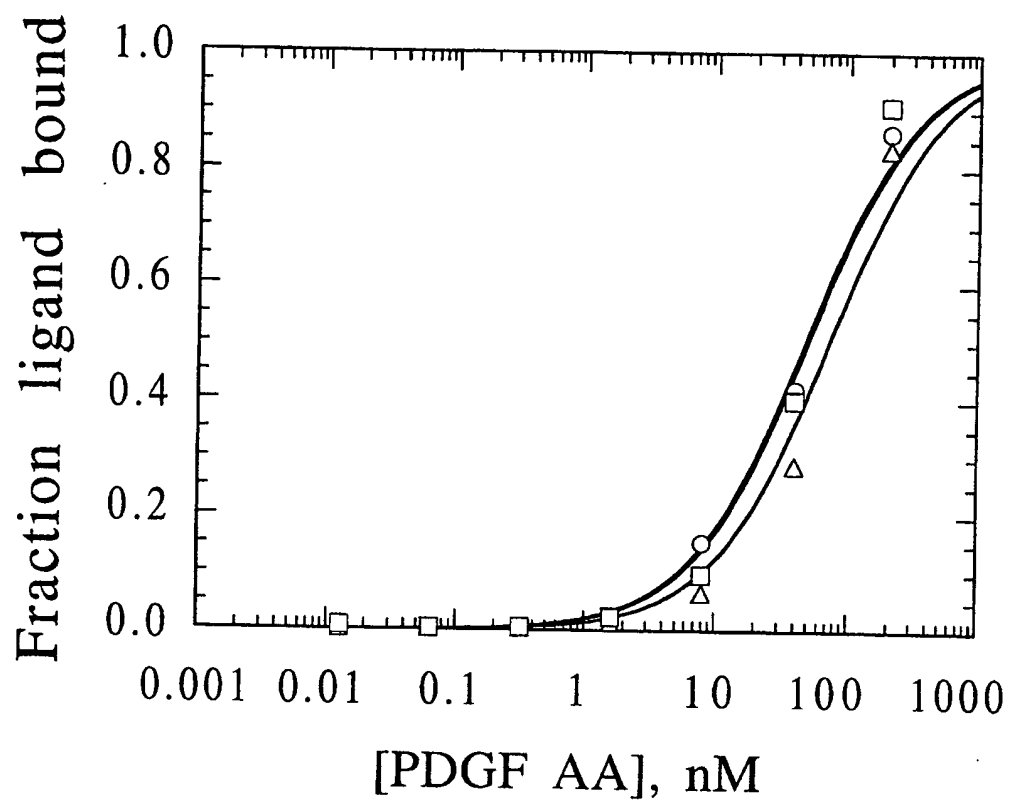


FIGURE 3A

4/26

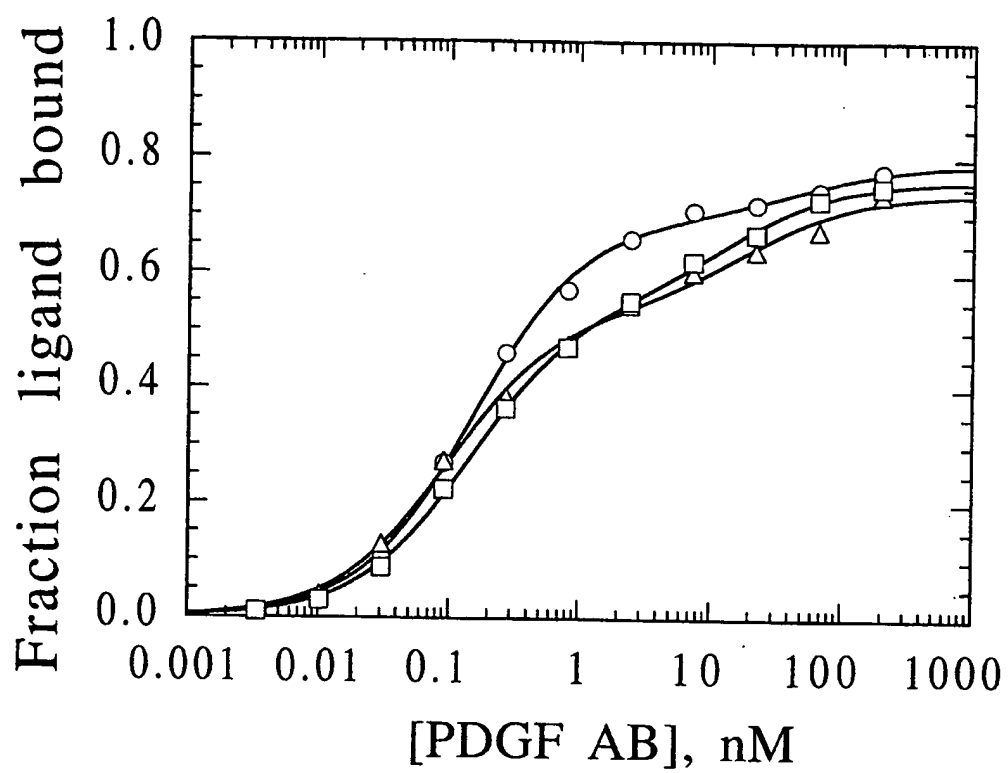


FIGURE 3B

5/26

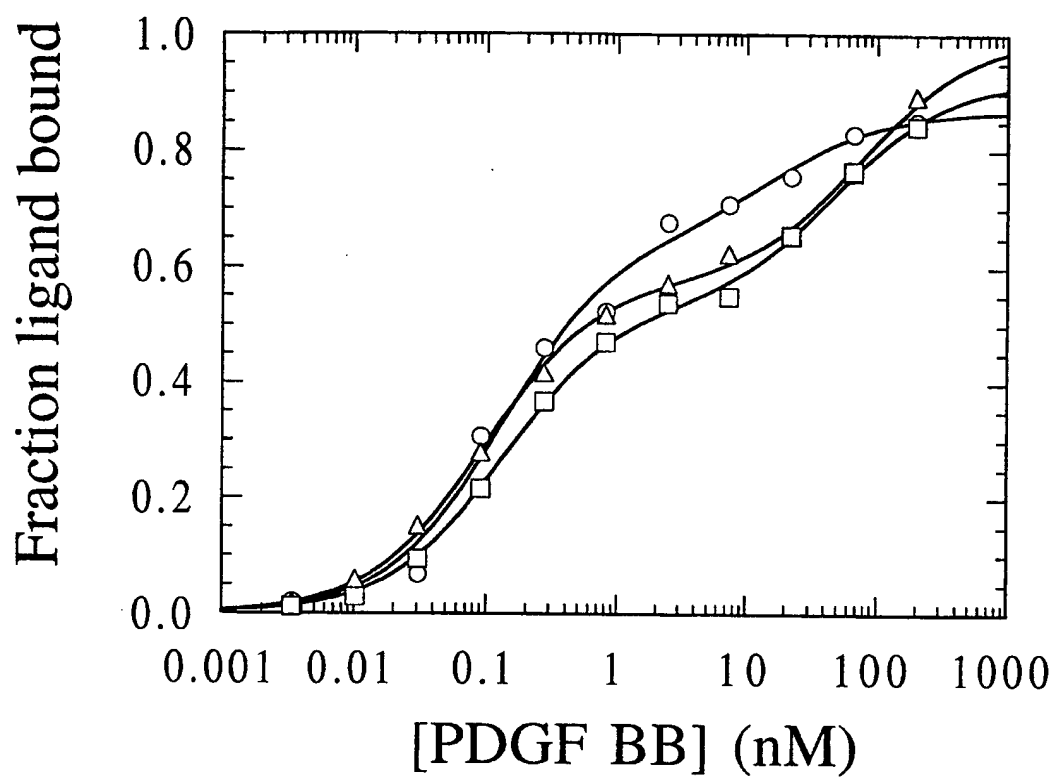


FIGURE 3C

6/26

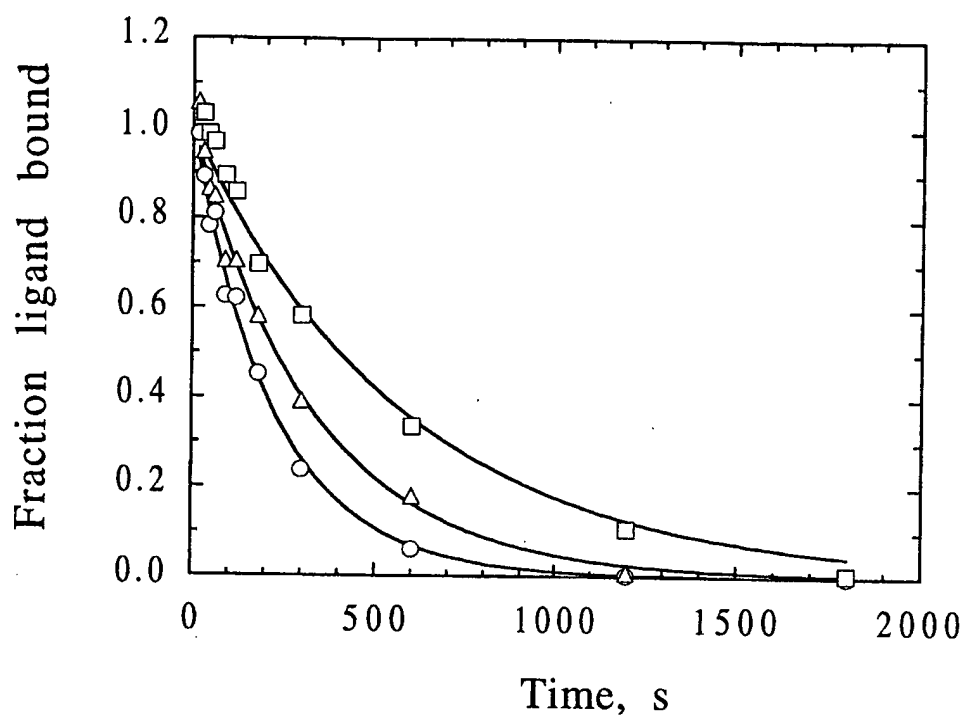


FIGURE 4

7/26

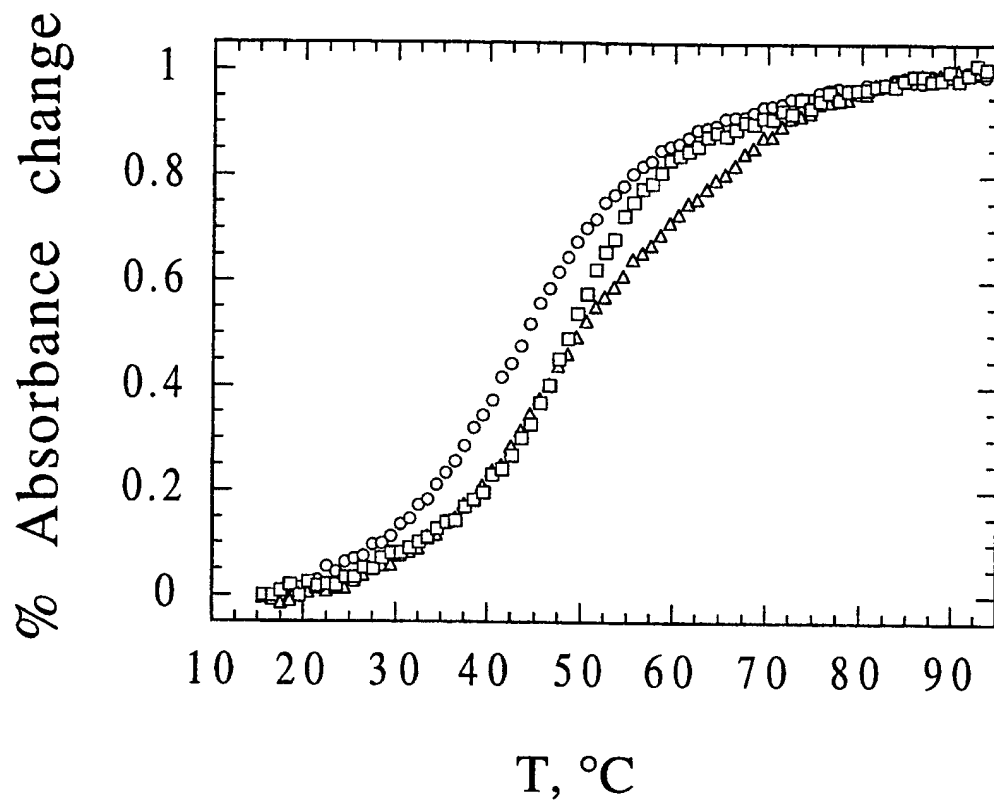


FIGURE 5

8/26

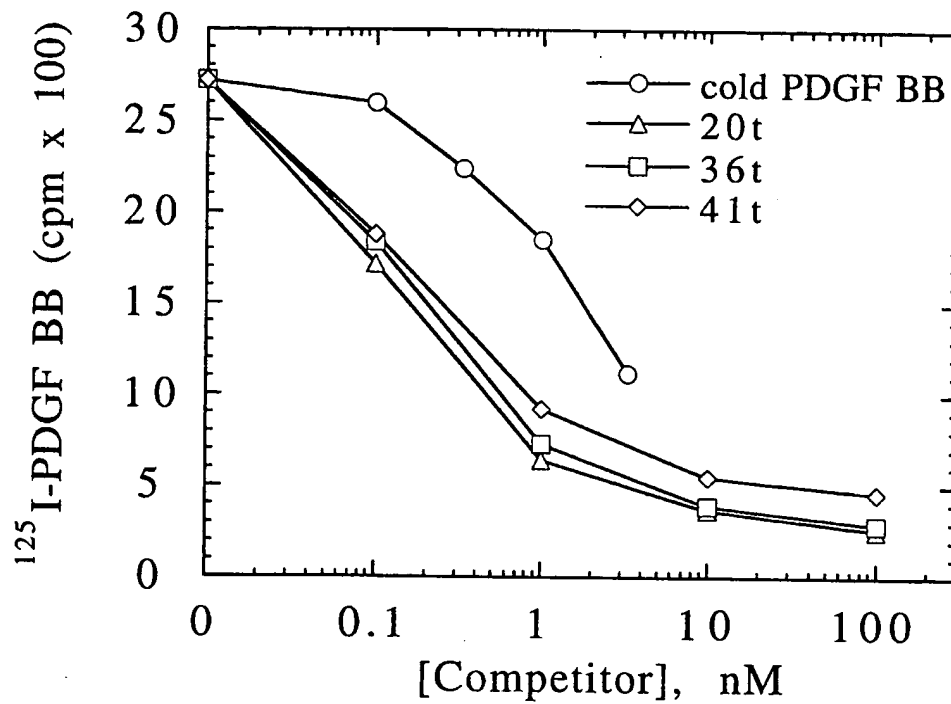


FIGURE 6

9/26

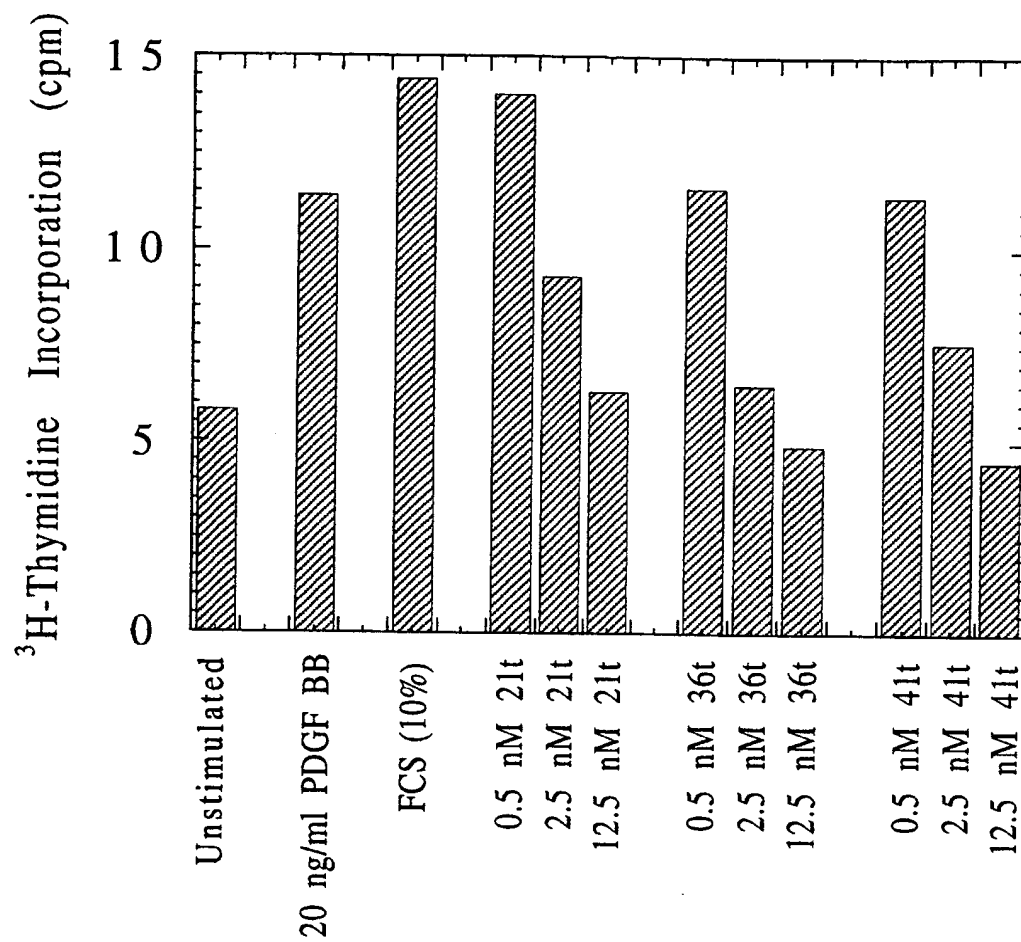


FIGURE 7



10/26

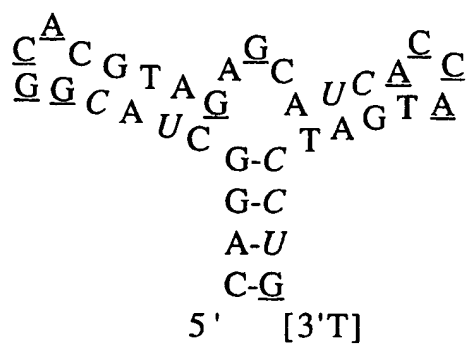


FIGURE 8A

 $K_d=0.065$  nM

SEQ ID NO: 86

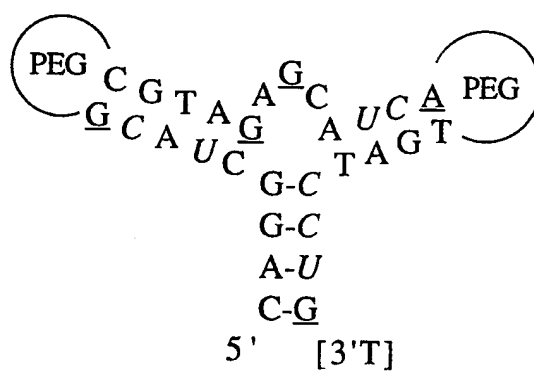


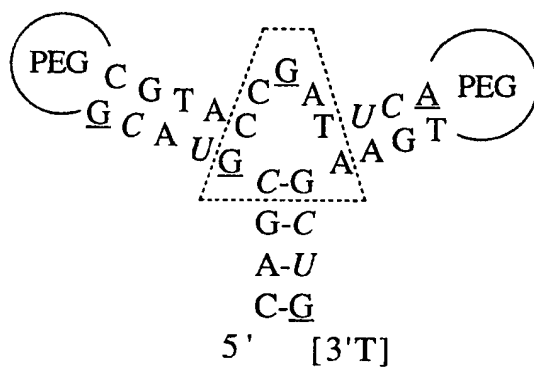
FIGURE 8B

 $K_d=0.097$  nM

NX21568

SEQ ID NO: 87

11/26



SEQ ID NO: 145

FIGURE 8C

**NX31975 40K PEG**  
**SEQ ID NO: 146**

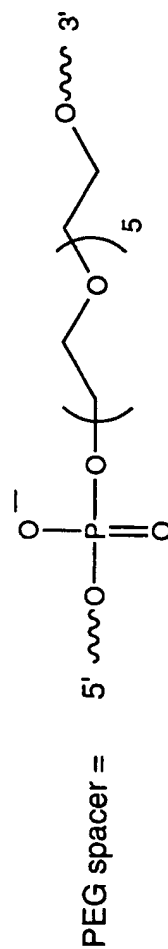
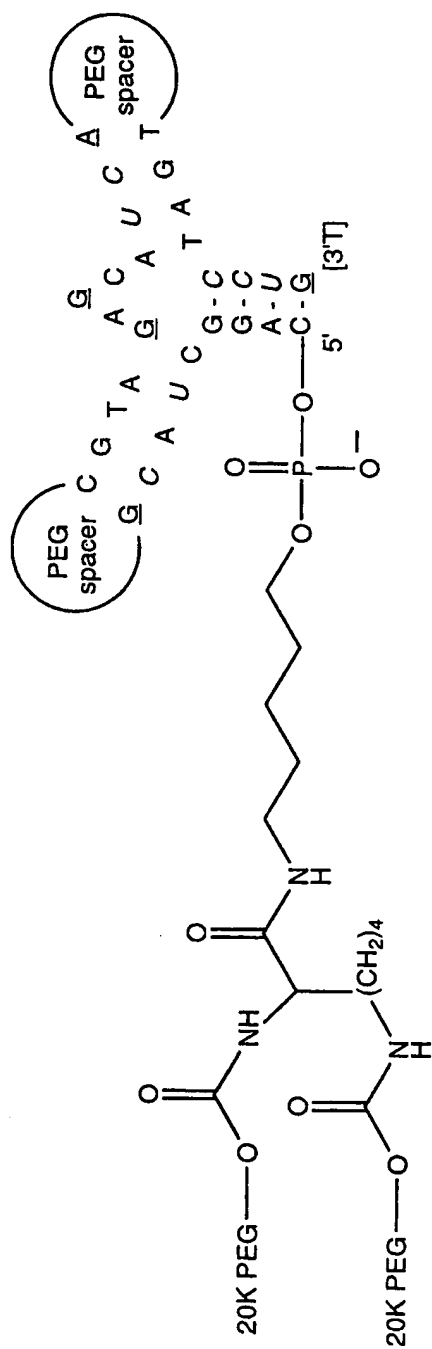
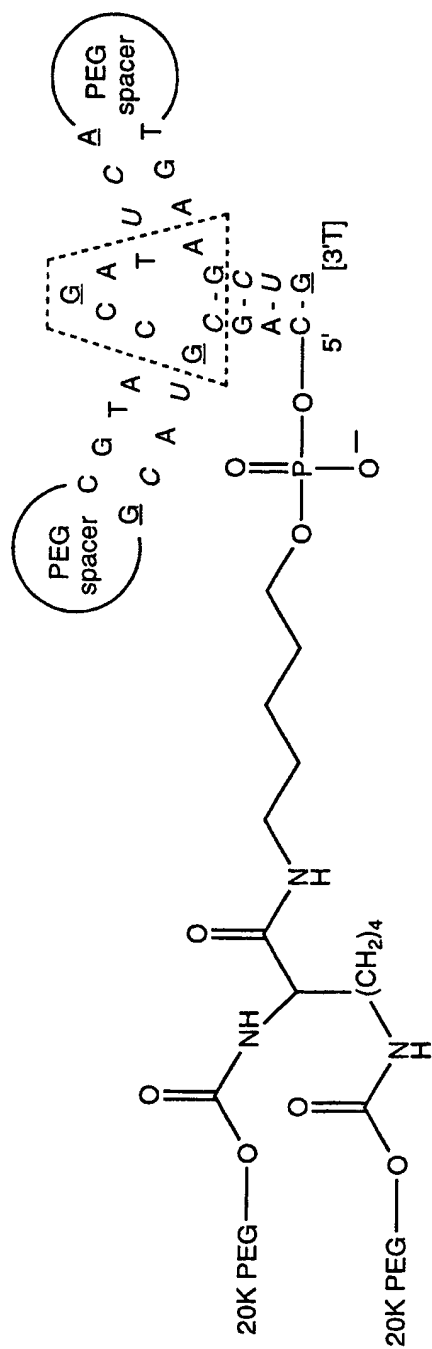


Figure 9A



NX31976 40K PEG  
SEQ ID NO: 147

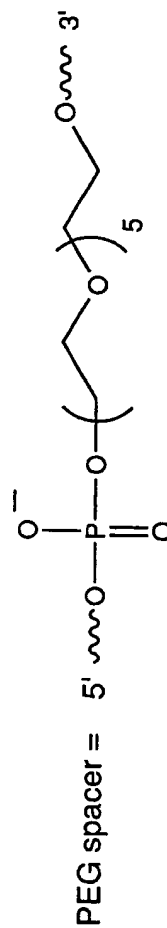


Figure 9B

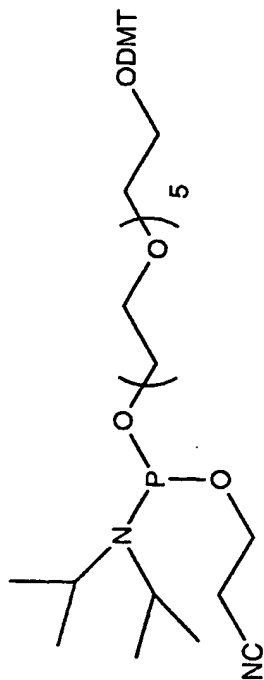


Figure 9C

Hexaethylene glycol spacer phosphoramidite

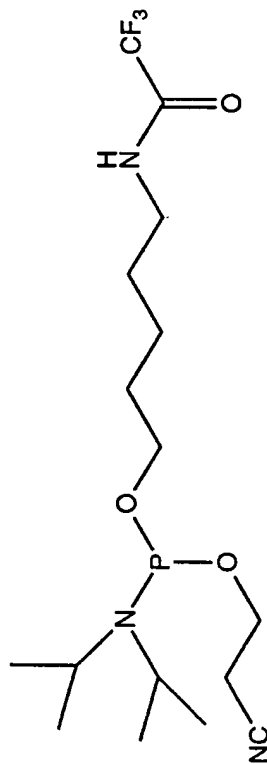
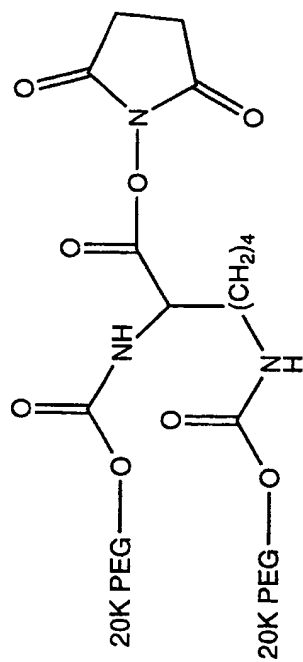


Figure 9D

Pentyl amino linker



40K PEG NHS ester

Figure 9E

16/26

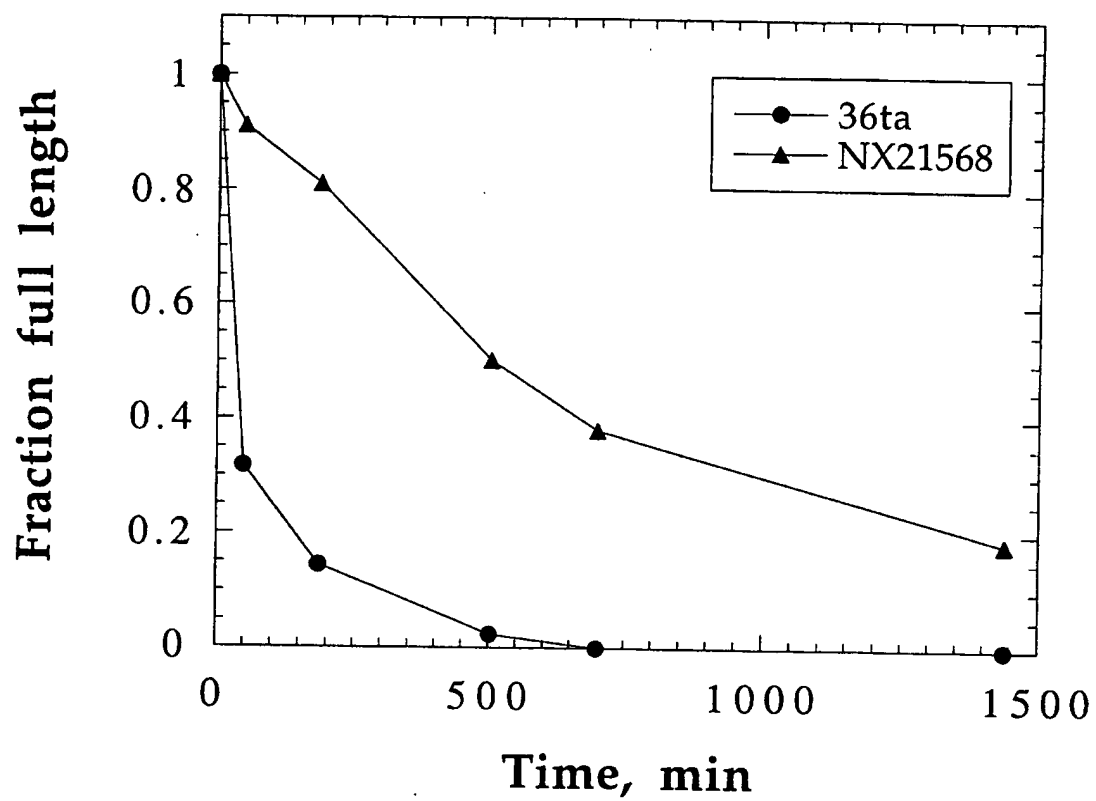


FIGURE 10

17/26

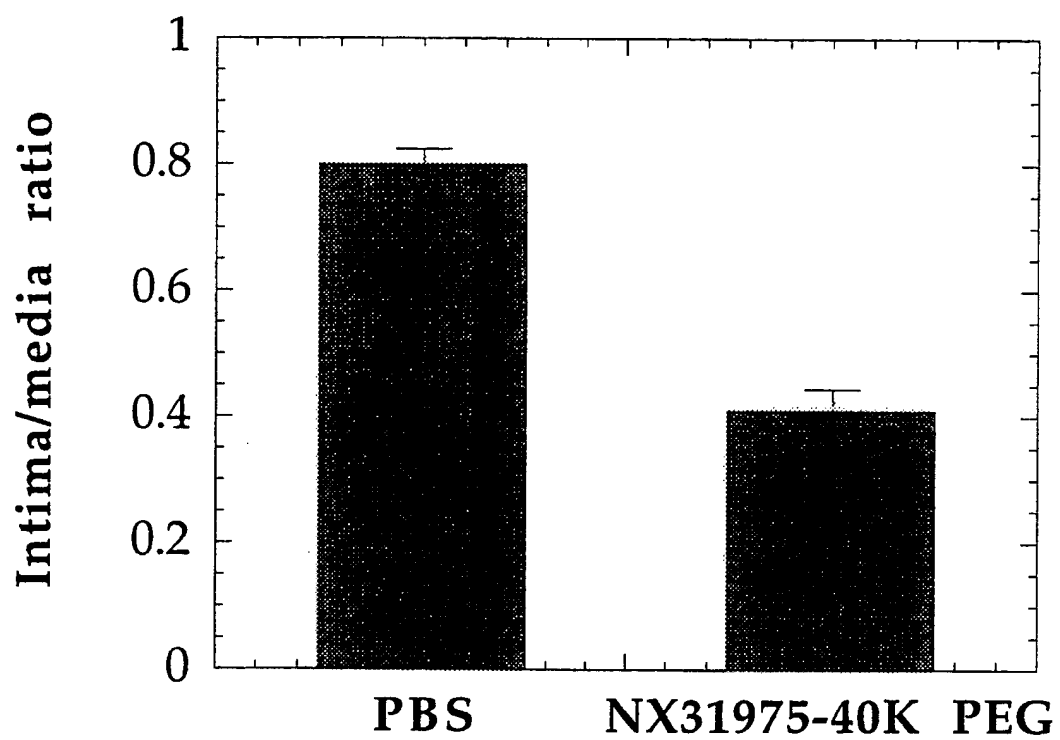


FIGURE 11



18/26

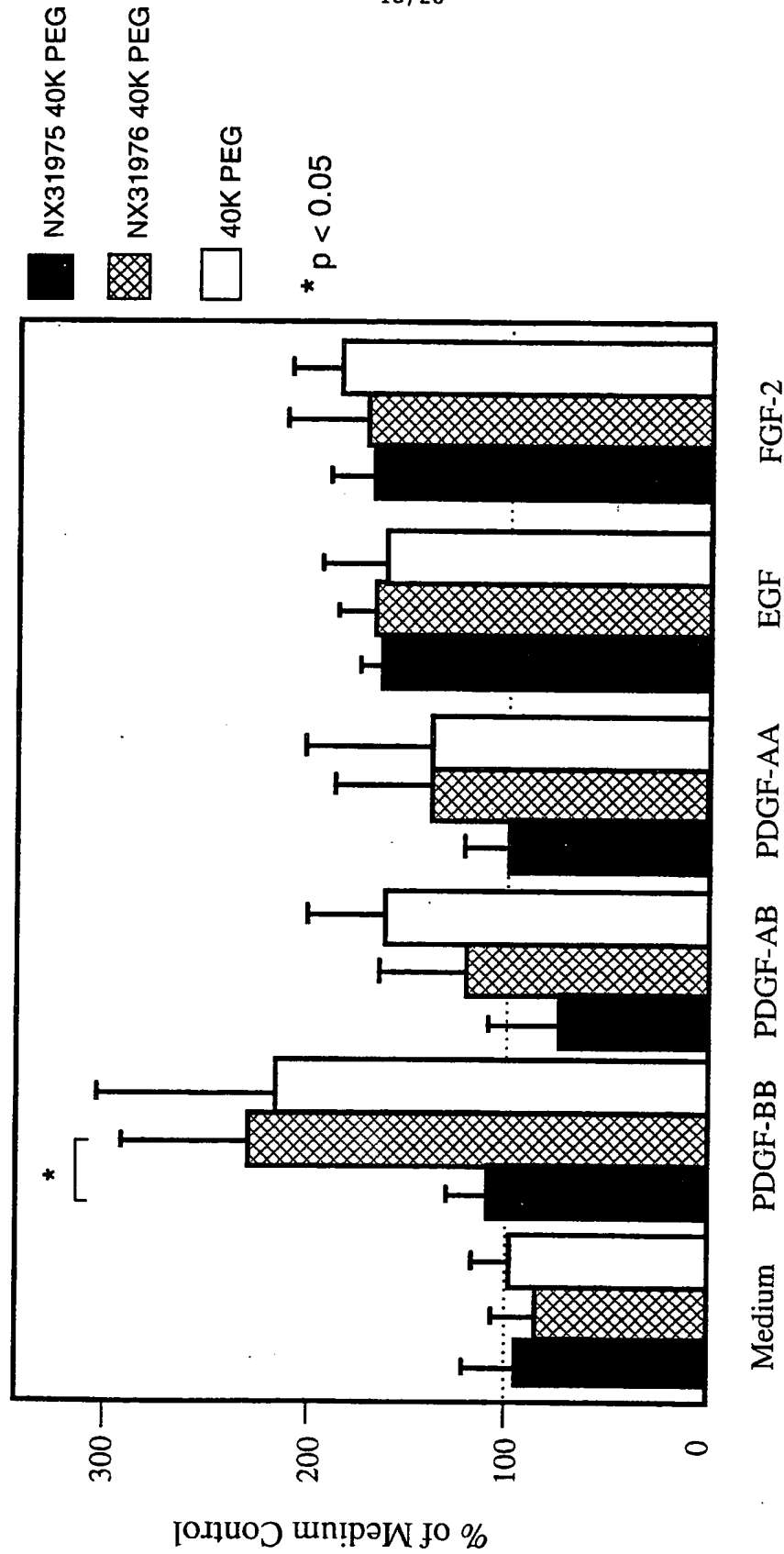


Figure 12

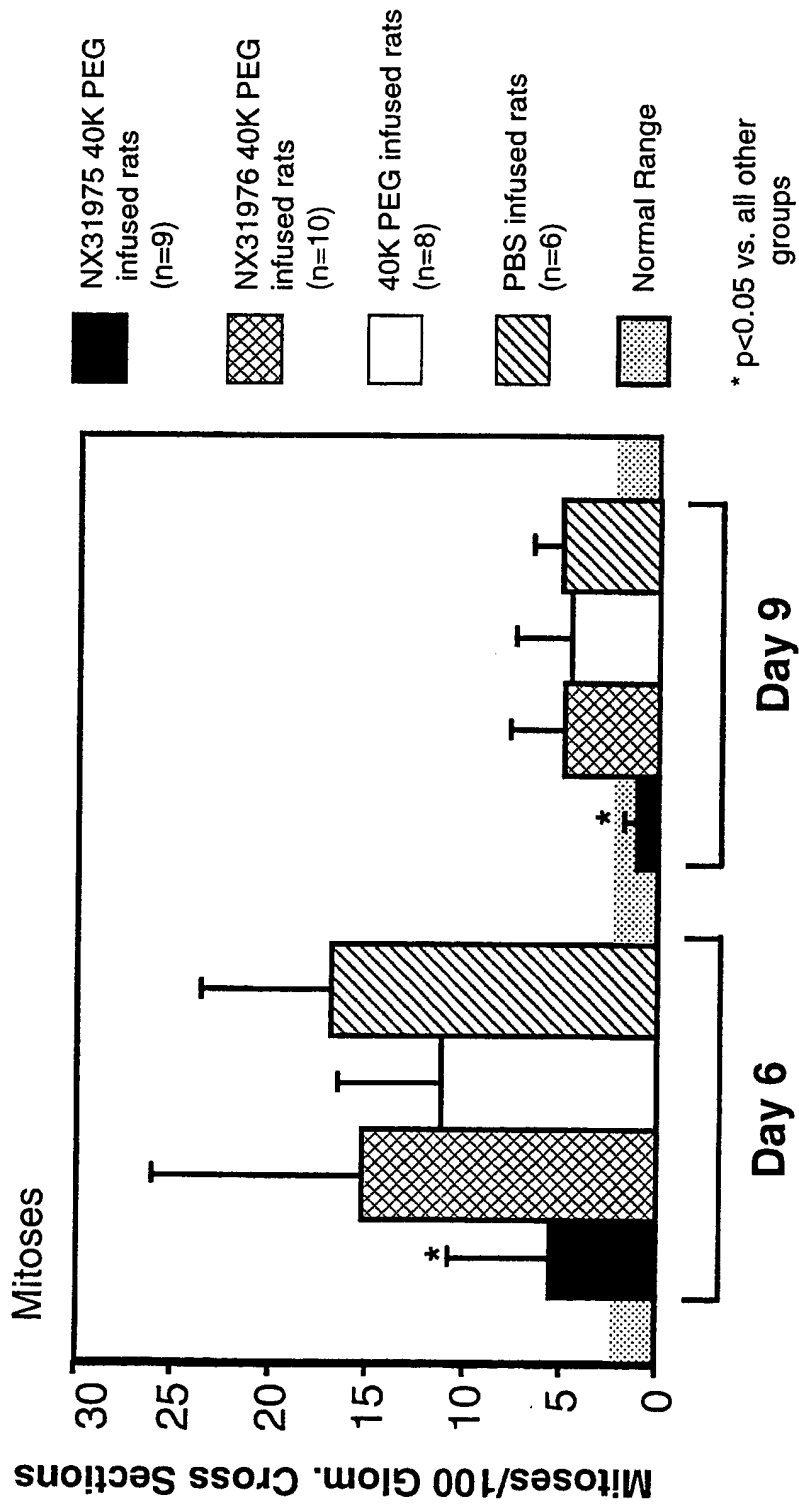


Figure 13A

20/26

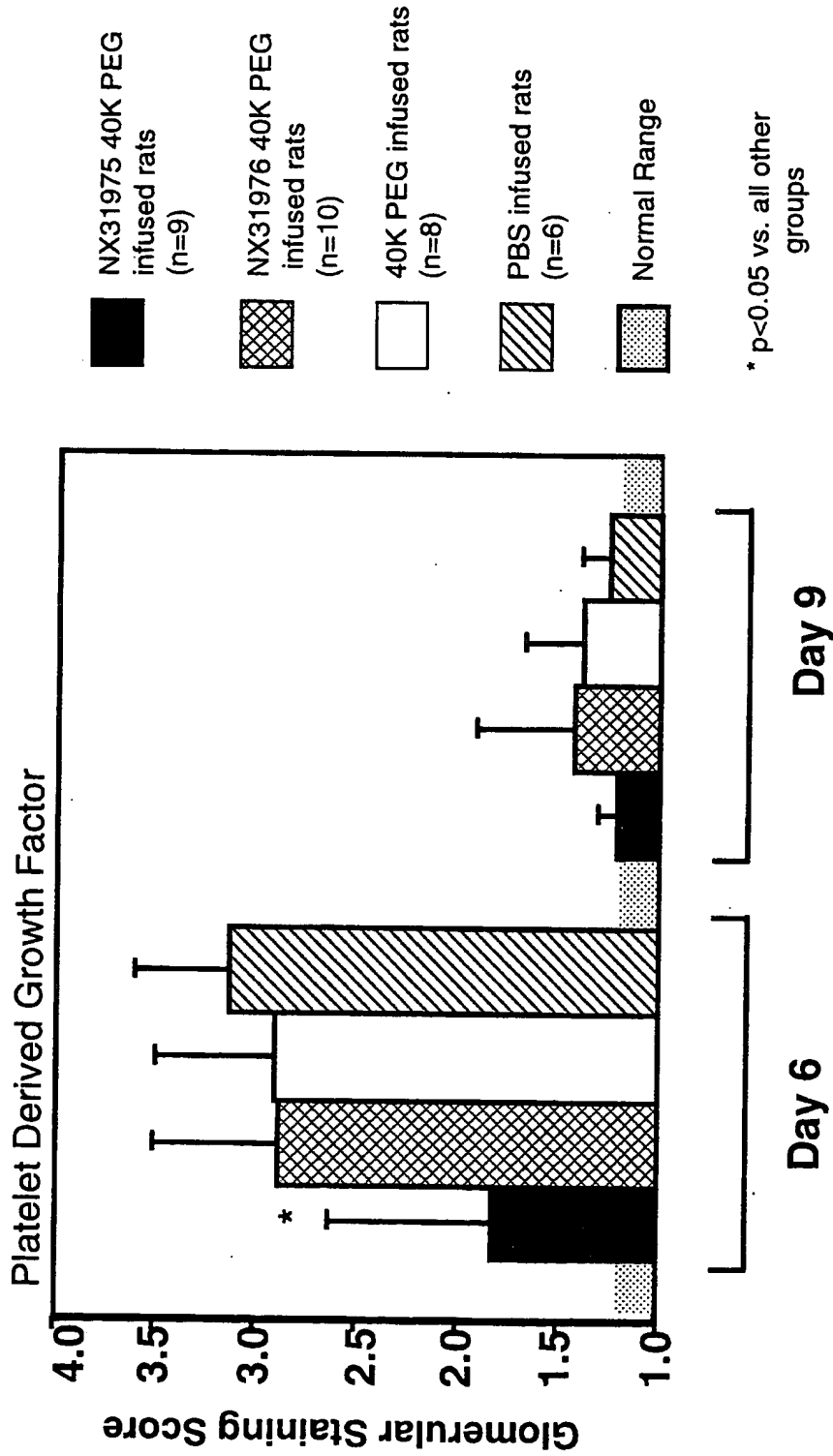


Figure 13B

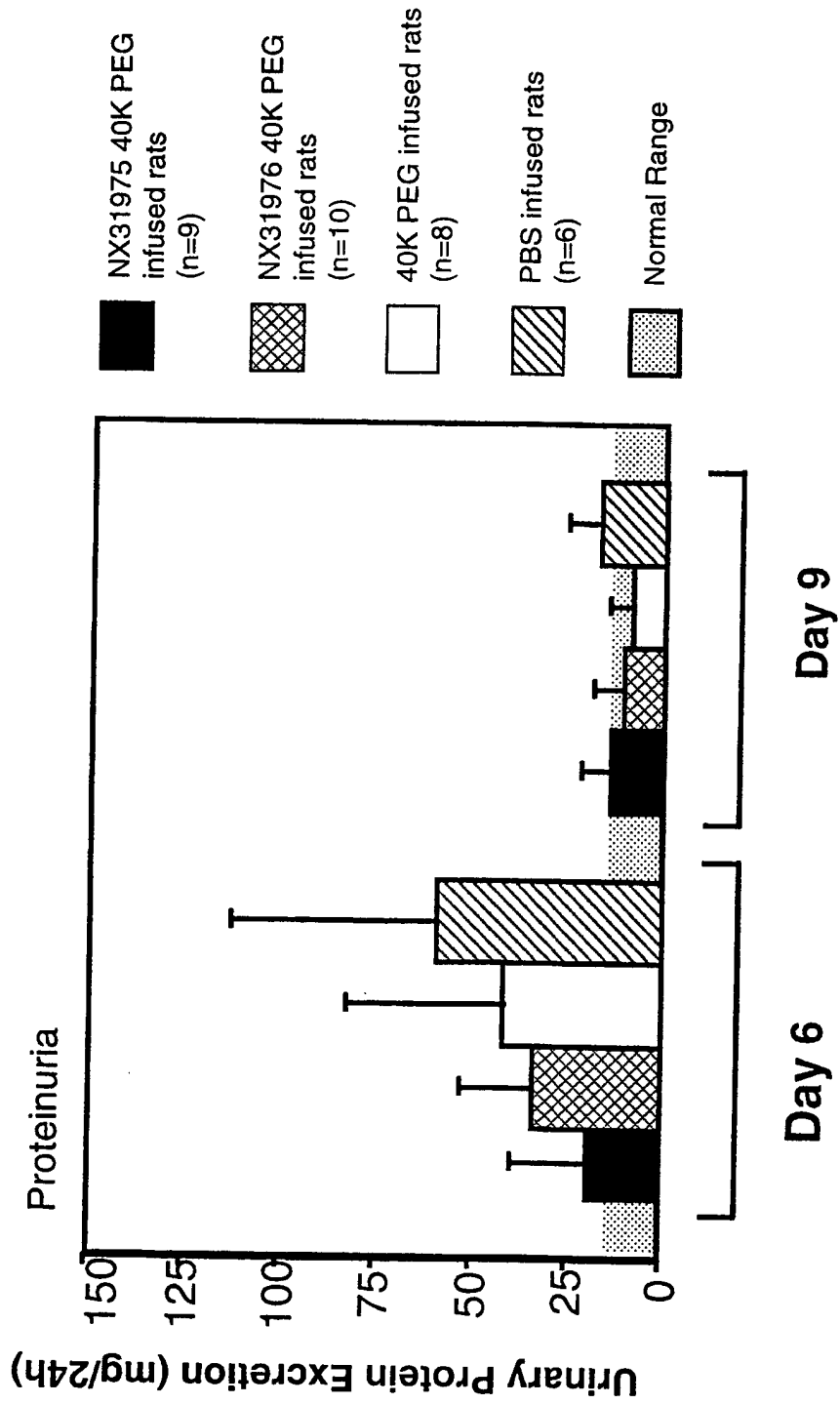


Figure 13C

22/26

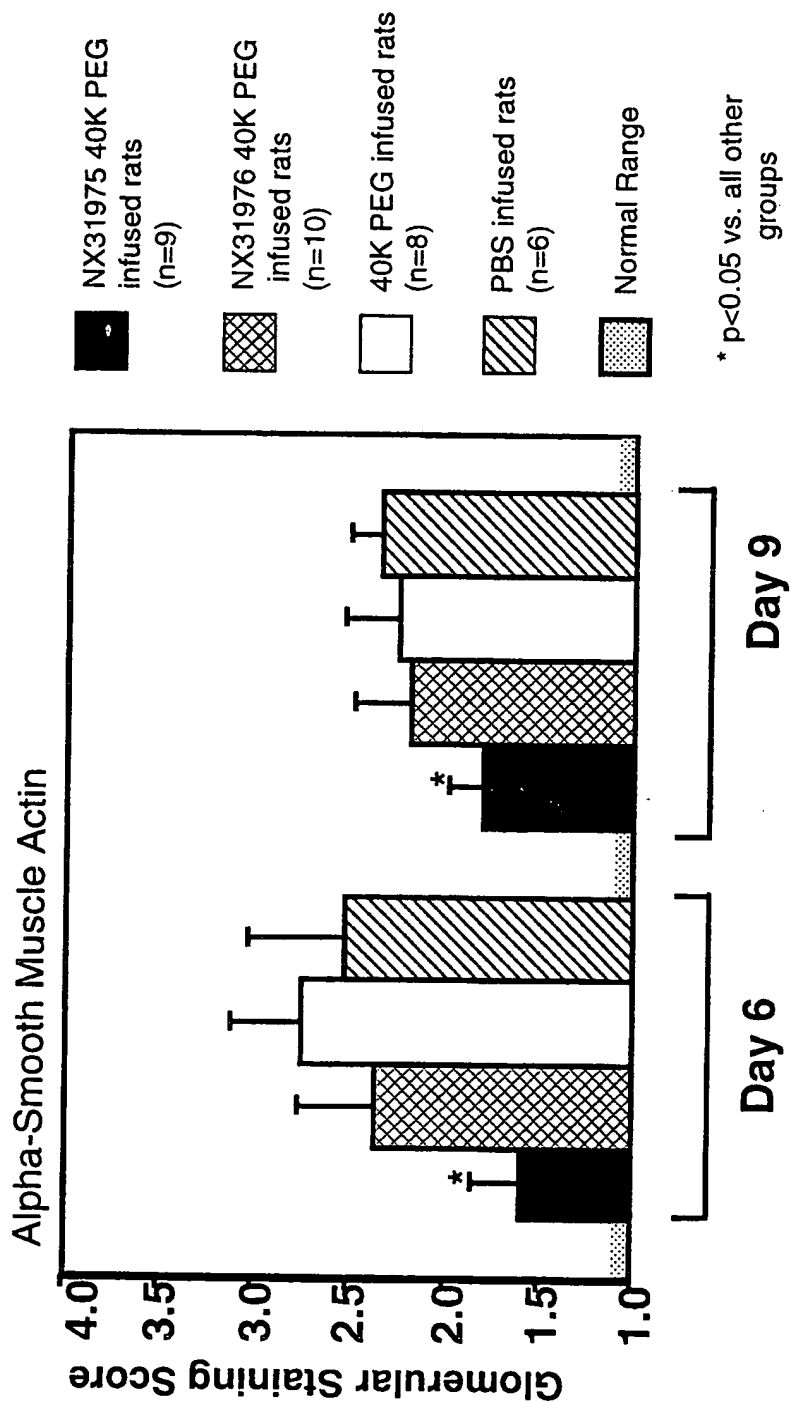


Figure 13D

23/26

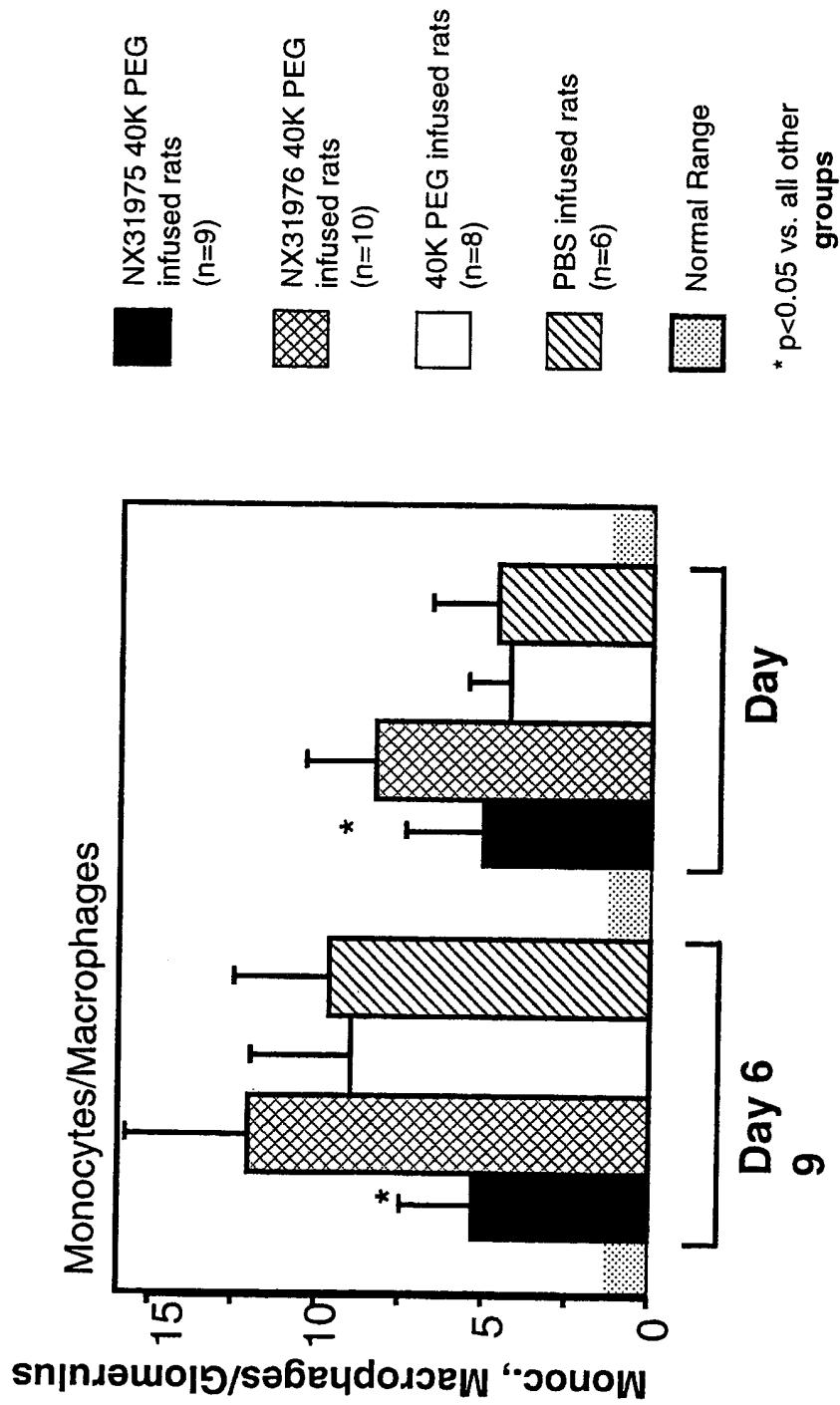
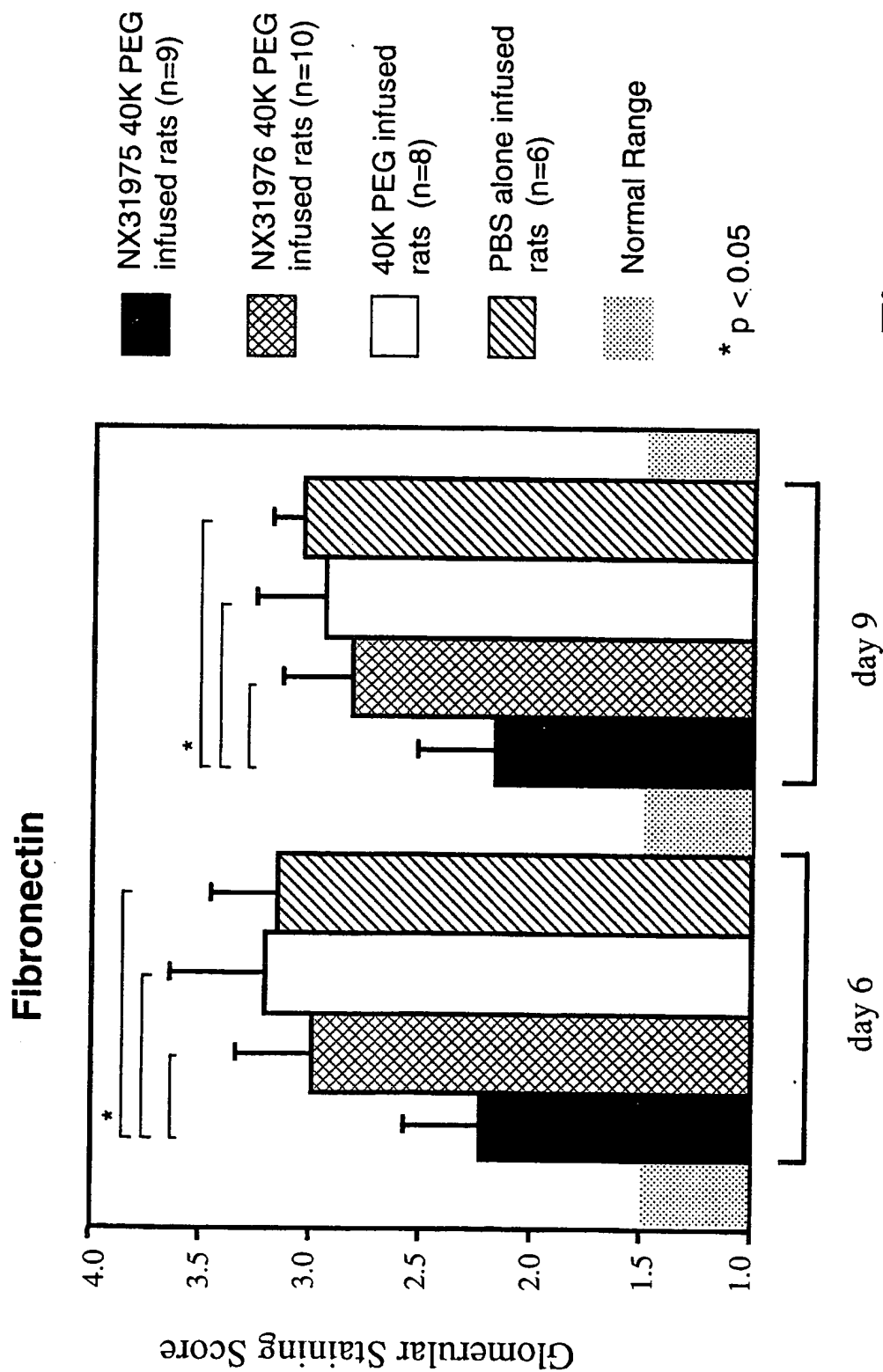


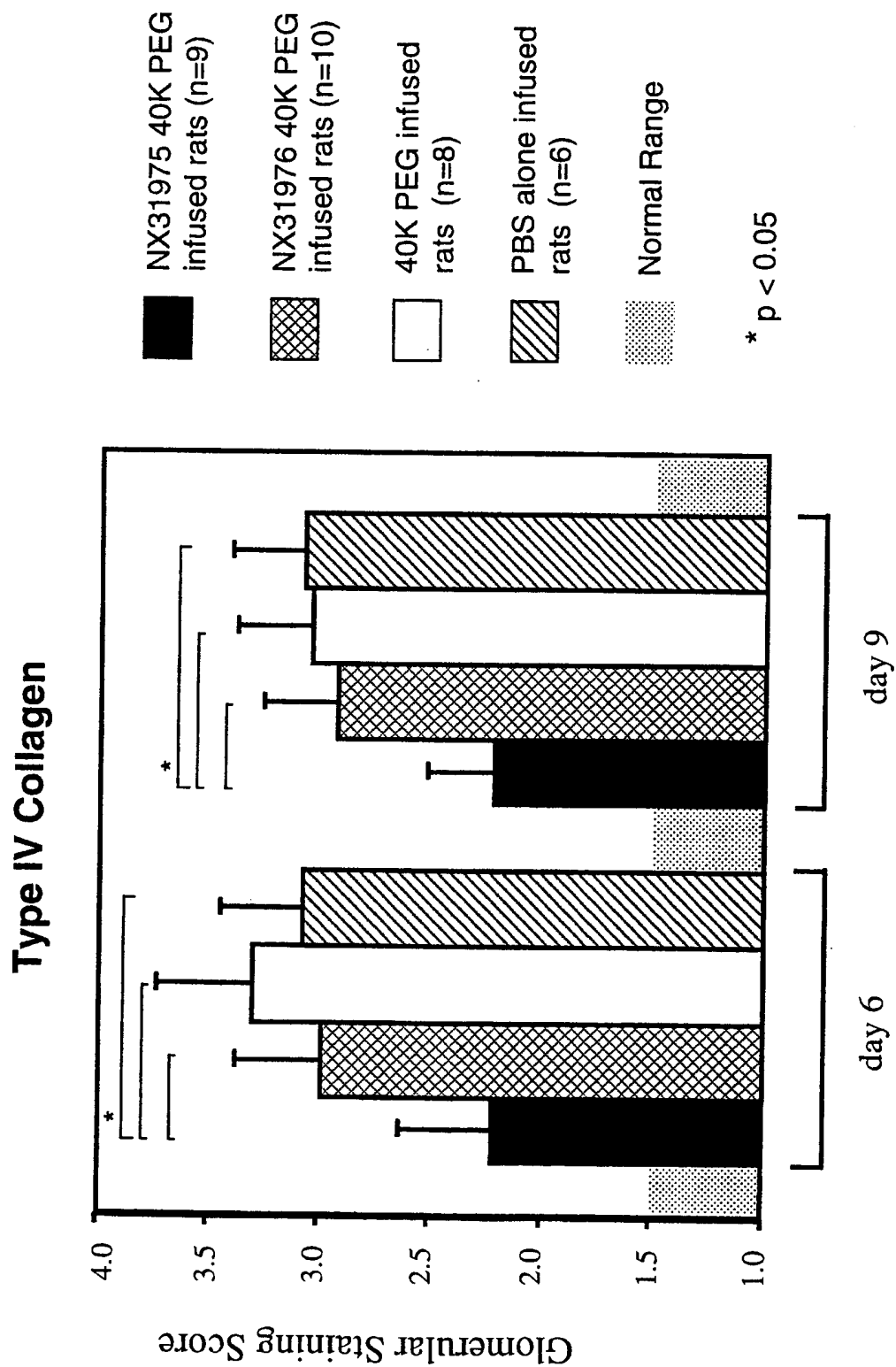
Figure 13E

24/26

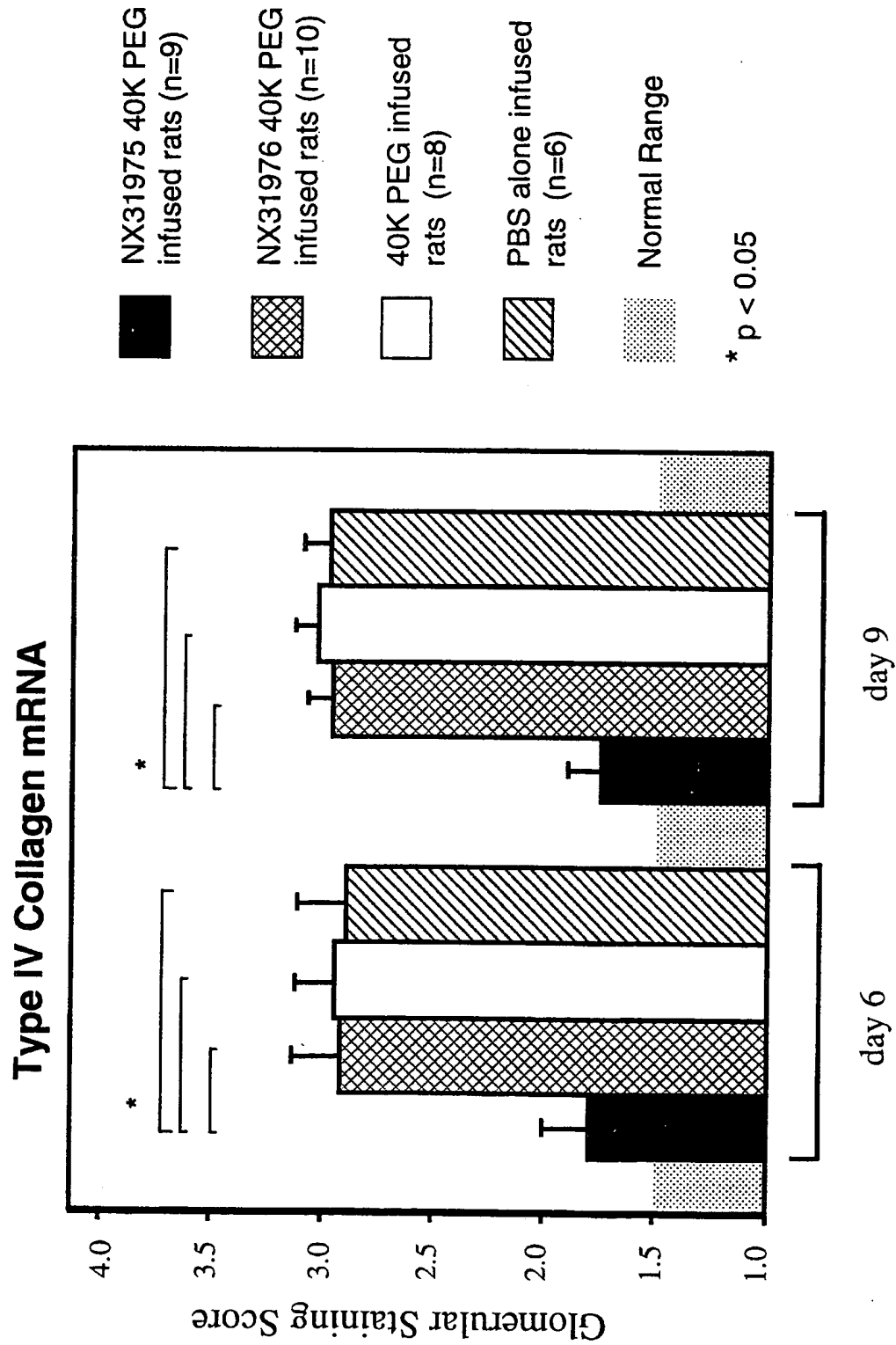


**Figure 14A**

25/26

**Figure 14B**





**Figure 14C**

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/09050

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : CO7H 21/04, 21/02; C12Q 1/68; C12P 19/34

US CL : 536/23.1; 435/6, 435/91.2

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.1; 435/6, 435/91.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN

search terms: platelet derived growth factor, PDGF, nucleic acid, lipophilic, selex

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	US 5,674,685 A (JANJIC et al) 07 October 1997, see entire document.	1, 2, 10-14, 22-27
X,P --- A,P	US 5,723,594 A (JANJIC et al.) 03 March 1998, see entire document.	1-7, 10-19, 22-28 ----- 8-9, 20 and 21

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*B* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 10 JULY 1998	Date of mailing of the international search report 08 SEP 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer <i>Christina Lawrence For</i> JEZIA RILEY Telephone No. (703) 308-0196

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/09050**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  
1-28

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

**BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING**

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-28, drawn to a complex of PDGF nucleic acid ligand, a method of treating a PDGF disease, and a method for the preparation of a complex PDGF nucleic acid.

Group II, claim(s) 29, drawn to a method for improving the pharmacokinetic properties of a PDGF nucleic acid ligand.

Group III, claim(s) 30, drawn to a method for targeting a therapeutic agent.

Group IV, claim(s) 31, drawn to method of inhibiting PDGF mediated angiogenesis.

Group V, claim(s) 32-34, drawn to a method of inhibiting the growth of tumors.

Group VI, claim(s) 35-46, drawn to a method of inhibiting fibrosis.

Group VII, claim(s) 47-57, drawn to a method of inhibiting restenosis.

The inventions listed as Groups I-VII do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Invention I correspond to one special technical feature (the complex of PDGF nucleic acid ligand), a method of making it and a method of using it. Inventions II-VII correspond to other methods of using said special technical feature. This application contains claims to more than one combination of categories of invention set forth in paragraph 1.475 (b) therefore unity of invention is lacking.

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**

